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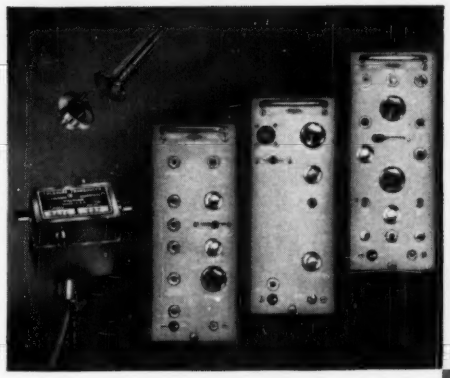

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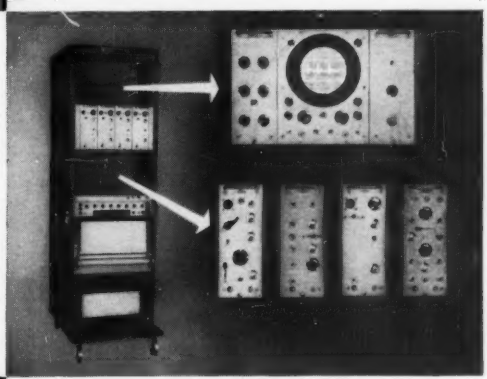

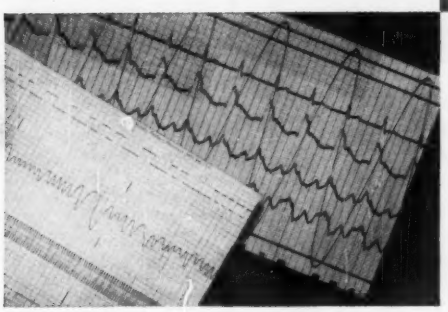

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3.

4.

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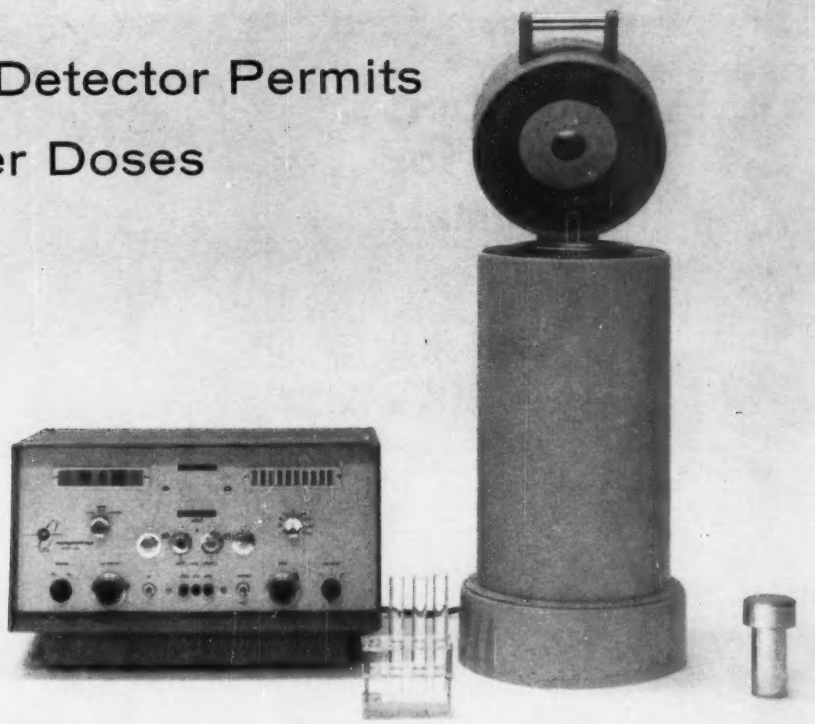

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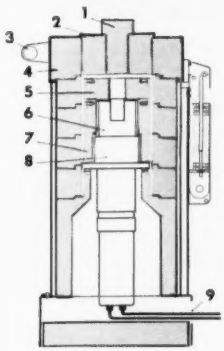

Cross-section of DS-303 Well Detector

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2. Removable Plug
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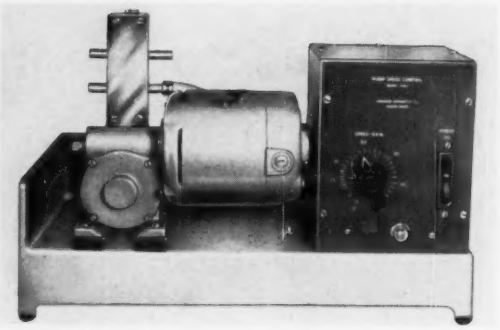

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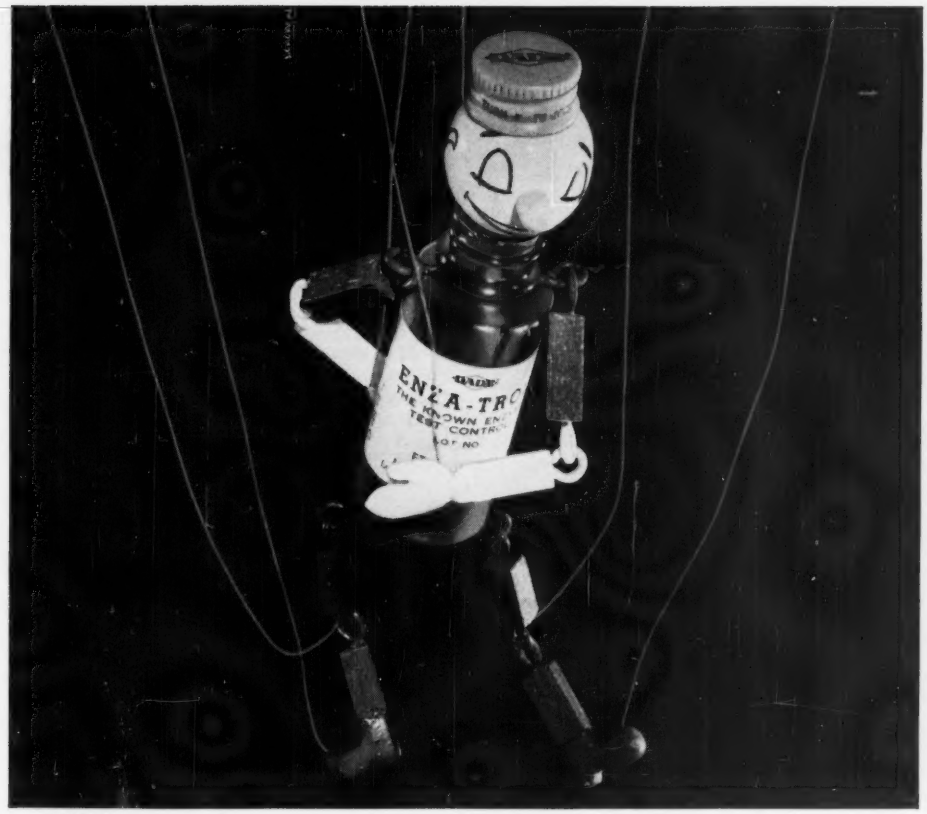

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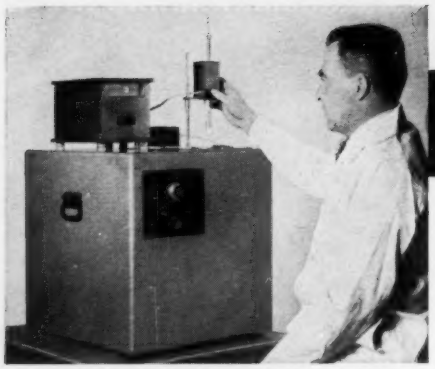

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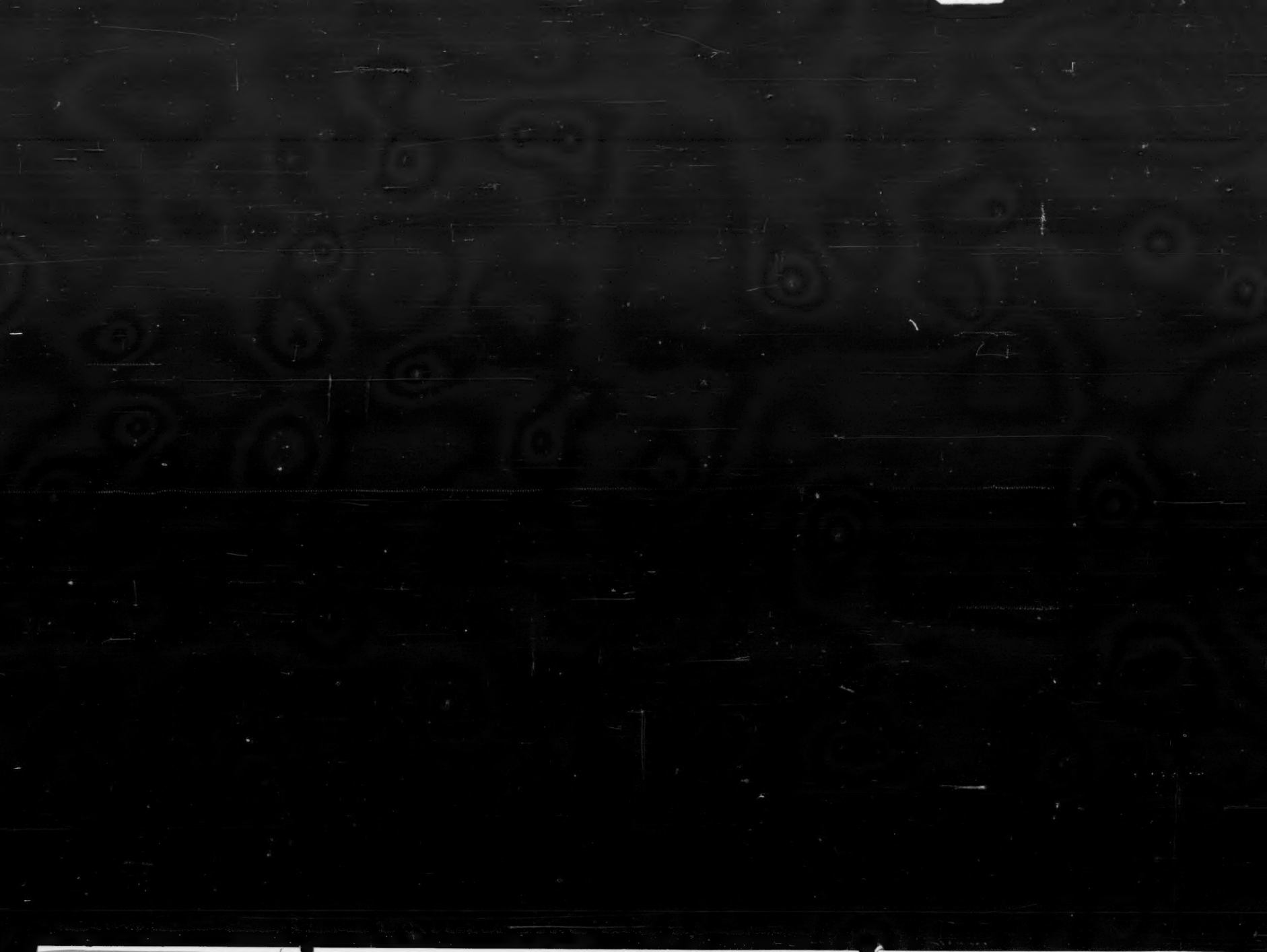

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Each dry filled vial contains: 1 Gm. STAPHCILLIN (sodium dimethoxyphenyl penicillin), equivalent to 900 mg. dimethoxyphenyl penicillin activity.

INDICATIONS

STAPHCILLIN is recommended as specific therapy only in infections due to strains of staphylococci resistant to other penicillins, e.g.:

Skin and soft tissue infections: cellulitis, wound infections, carbuncles, pyoderma, furunculosis, lymphangitis and lymphadenitis.

Respiratory infections: staphylococcal lobar or bronchopneumonia, and lung abscesses combined with indicated surgical treatment.

Other infections: staphylococcal septicemia, bacteremia, acute or subacute endocarditis, acute osteomyelitis and enterocolitis.

Infections due to penicillin-sensitive staphylococci, streptococci, pneumococci and gonococci should be treated with Syncillin® or parenteral penicillin G rather than STAPHCILLIN. Treponemal infections should be treated with parenteral penicillin G.

DOSAGE AND ADMINISTRATION

STAPHCILLIN is well tolerated when given by deep intragluteal or intravenous injection.

As is the case with other antibiotics, the duration of therapy should be determined by the clinical and bacteriological response of the patient. Therapy should be continued for at least 48 hours after the patient has become afebrile, asymptomatic and cultures are negative. The usual duration has been 5-7 days.

Intramuscular route: The usual adult dose is 1 Gm. every 4 or 6 hours. Infants' and children's dosage is 25 mg. per Kg. (approximately 12 mg. per pound) every 6 hours.

Intravenous route: 1 Gm. every 6 hours using 50 ml. of sterile saline solution at the rate of 10 ml. per minute.

**Warning:* Solutions of STAPHCILLIN and kanamycin should not be mixed, as they rapidly inactivate each other. Data on the results of mixing STAPHCILLIN with other antibiotics are being accumulated.

DIRECTIONS FOR RECONSTITUTION

Add 1.5 ml. sterile distilled water or normal saline to a 1 Gm. vial and shake vigorously. Withdraw the clear, reconstituted solution (2.0 ml.) into a syringe and inject. The reconstituted solution contains 500 mg. of STAPHCILLIN per ml. Reconstituted solutions are stable for 24 hours under refrigeration.

For intravenous use, dilute the reconstituted dose in 50 ml. of sterile saline and inject at the rate of 10 ml. per minute.

*This statement supersedes that in the Official Package Circulars dated September and/or October, 1960.

(continued)

MICROBIOLOGICAL AND PHARMACOLOGICAL PROPERTIES

In vitro studies show that STAPHCILLIN is a bactericidal penicillin with activity against staphylococci resistant to penicillin G. Strains of staphylococci so far tested have been sensitive to STAPHCILLIN *in vitro* at concentrations of 1-6 mcg. per ml. These levels are readily attained in the blood and tissues by administration of STAPHCILLIN at the recommended dosage. This unique attribute is probably due to the fact that STAPHCILLIN is stable in the presence of staphylococcal penicillinase. STAPHCILLIN also resists degradation by *B. cereus* penicillinase. The antomicrobial spectrum of STAPHCILLIN with regard to other microorganisms is qualitatively similar to that of penicillin G; but considerably higher concentrations of STAPHCILLIN are required for bactericidal activity than is the case with penicillin G.

STAPHCILLIN is rapidly absorbed after intramuscular injection. Peak blood levels (6-10 mcg./ml. on the average after a 1.0 Gm. dose) are attained within 1 hour; and then progressively decline to less than 1 mcg. over a 4 to 6 hour period. It is poorly absorbed from the gastrointestinal tract. STAPHCILLIN is rapidly excreted by the kidney.

As shown by animal studies, STAPHCILLIN is readily distributed in body tissues after intramuscular injection. Of the tissues studied, highest concentrations are reached in the kidney, liver, heart and lung in that order; the spleen and muscles show lower concentrations of the antibiotic. STAPHCILLIN diffuses into human pleural and prostatic fluids, but its diffusion into the spinal fluid has not yet been completely studied. However, one patient with meningitis showed a significant concentration in his spinal fluid while on STAPHCILLIN therapy.

Toxicity studies with STAPHCILLIN and penicillin G in animals show that they have approximately the same low order of toxicity.

Certain staphylococci can be made resistant to STAPHCILLIN in the laboratory, but this resistance is not related to their penicillinase production. During the clinical trials, no STAPHCILLIN-resistant strains of staphylococci were observed or developed; the possibility of the emergence of such strains in the clinical setting awaits further observation.

PRECAUTIONS

During the clinical trials, several mild skin reactions, e.g., itching, papular eruption and erythema were observed both during and after discontinuance of STAPHCILLIN therapy. Patients with histories of hay fever, asthma, urticaria and previous sensitivity to penicillin are more likely to react adversely to the penicillins. It is important that the possibility of penicillin anaphylaxis be kept in mind. Epinephrine and the usual adjuvants (antihistamines, corticosteroids) should be available for emergency treatment. Because of the resistance of STAPHCILLIN to destruction by penicillinase, parenteral *B. cereus* penicillinase may not be effective for the treatment of allergic reactions. Information with regard to cross-allergenicity between penicillin G, penicillin V, phenethicillin (Syncillin) and STAPHCILLIN is not available at present. If superinfection due to Gram-negative organisms or fungi occurs during STAPHCILLIN therapy, appropriate measures should be taken.

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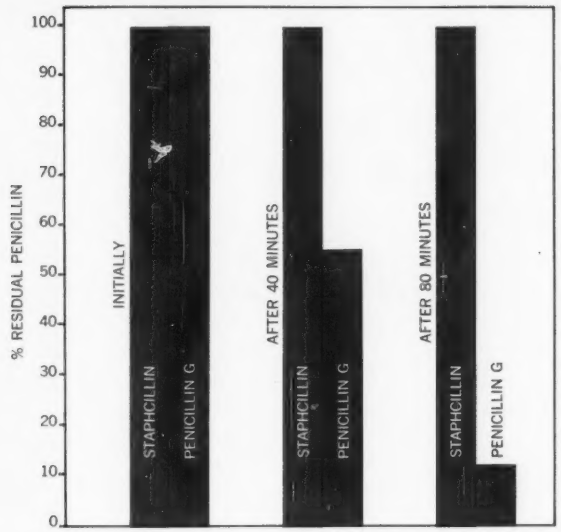

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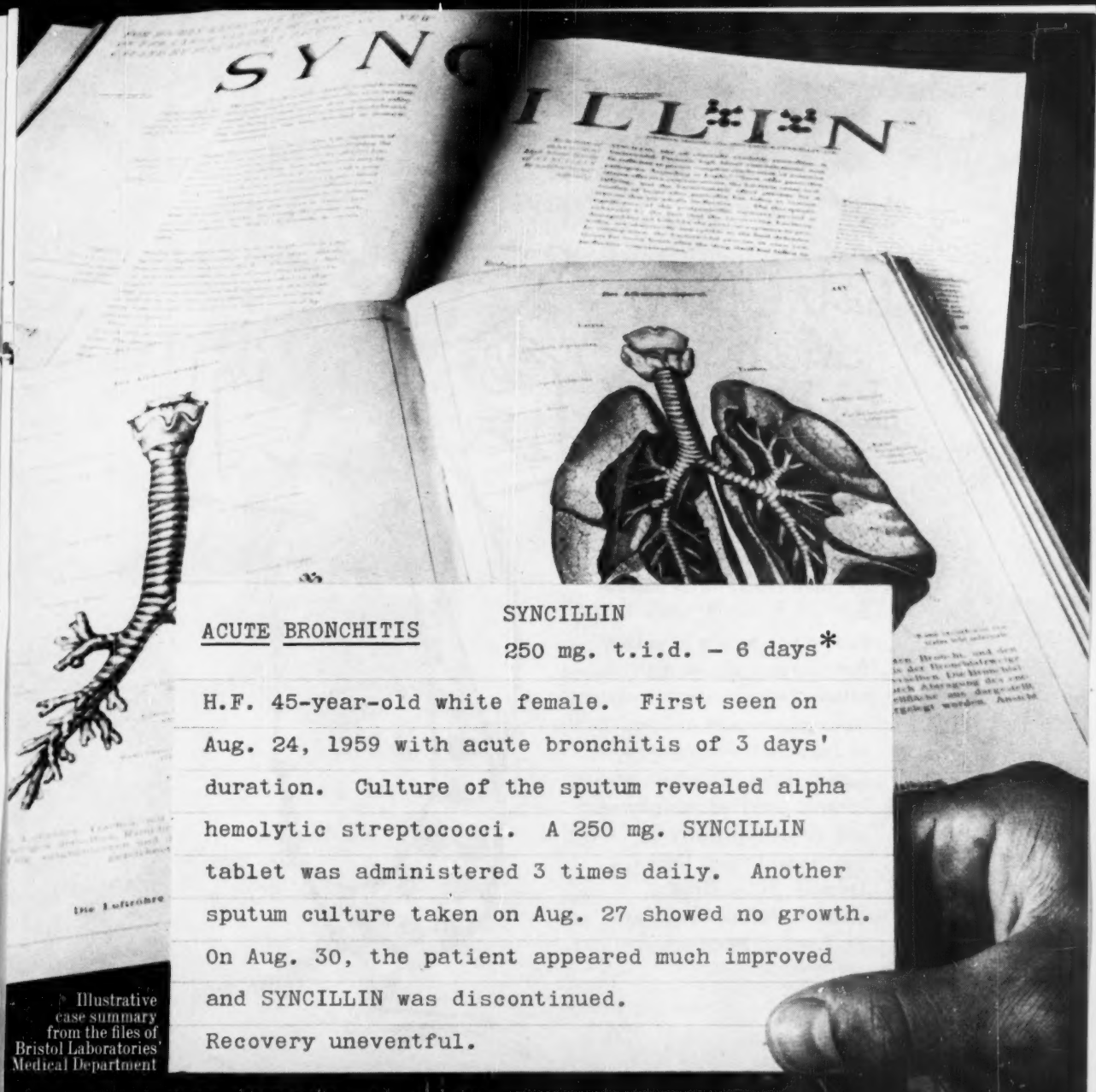

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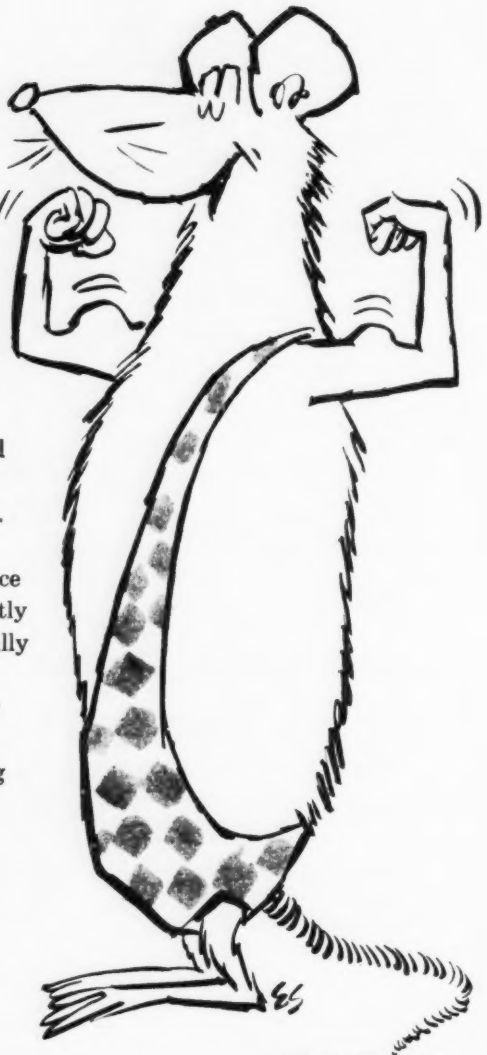

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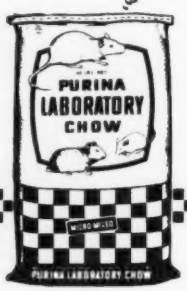

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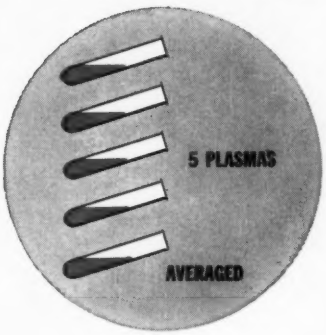

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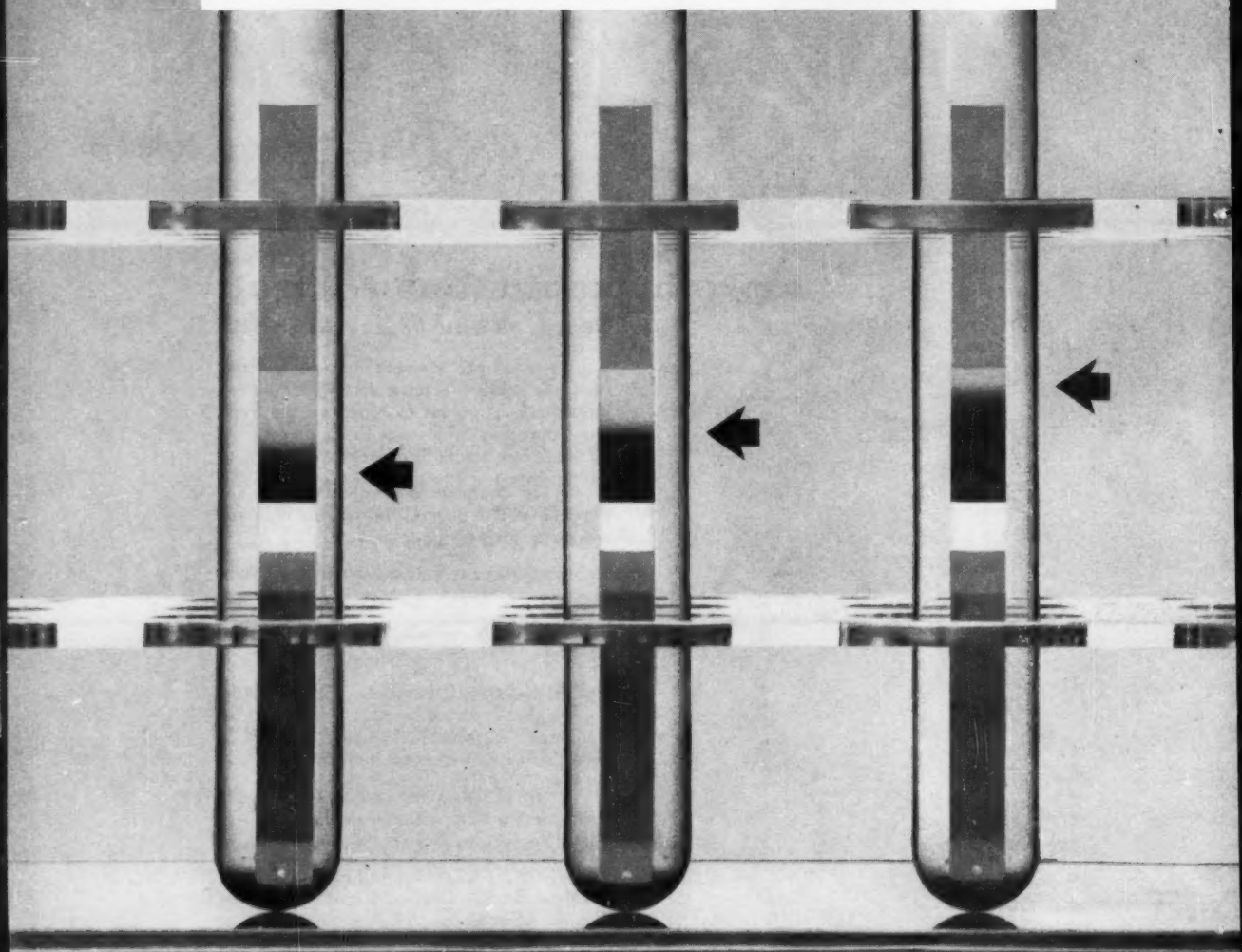

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AMINO ACID ACTIVATING ENZYMES IN RED BLOOD CELLS OF NORMAL, ANEMIC AND POLYCYTHEMIC SUBJECTS*

By G. IZAK, T. WILNER AND J. MAGER WITH THE TECHNICAL ASSISTANCE OF A. KARSHAI

(From the Department of Medicine B, and the Hematology Research Laboratory of Hadassah University Hospital and from the Department of Biochemistry, Hebrew University–Hadassah Medical School, Jerusalem, Israel)

(Submitted for publication May 18, 1960; accepted June 9, 1960)

Following the discovery by Hoagland (1) that the soluble cytoplasmic fraction of rat liver homogenates catalyzes an amino acid-dependent pyrophosphate (PP) exchange with adenosine triphosphate (ATP), similar systems have been described in a variety of other tissues and organisms (2–4). These so-called "amino acid activating enzymes" (AAAE) serve, according to the currently prevailing opinion (5), to convert free amino acids into the corresponding aminoacyl adenylates, thus initiating the chain of reactions mediating their incorporation into the protein molecule.

The present communication provides evidence for the occurrence of AAAE in human red blood cells (RBC). In addition, the paper presents quantitative data indicating the existence of some significant deviations from the normal level of activity of these enzymes in certain well defined blood disorders.

MATERIALS AND METHODS

Blood samples. Blood was obtained by venipuncture from healthy donors and anemic or polycythemic patients. The salient hematological data are recorded in Table I.

Preparation of hemolysates. The cells from freshly drawn heparinized blood samples were sedimented by centrifugation, washed 3 times with 2 vol of physiological saline buffered with veronal, pH 7.3, and counted before the last washing. All the subsequent operations were carried out in the cold.

Two ml of the packed blood cells were hemolyzed by dilution with 1.6 vol of cold distilled water. Following 10 minutes of stirring, the isotonicity of the hemolysate was restored by adding concentrated sucrose solution to a final concentration of 0.25 M, and the volume was made up to 10 ml. Ghosts and particles were removed by spinning the hemolysate for 30 minutes at 15,000 G in the Servall centrifuge. The resulting clear supernatant solution was carefully decanted and served as the source of amino acid activating enzymes. The hemolysate could be stored at $-15°$ C for at least 2 weeks without appreciable loss of activity with respect to amino acid activation. This preparation was routinely used and no attempt was made to exclude platelets and leukocytes, since the contribution of these cells to the over-all activity of the lysate was found to be negligible (see Results).

In some experiments the washed blood cells suspended in 0.25 M sucrose were disrupted by sonication in an ice water-cooled 10 KC-Raytheon oscillator.

Chemicals. All chemicals were of analytic or reagent grade. The radioactive pyrophosphate was prepared by pyrolysis of P^{32}-labeled orthophosphate at 280° C for 20 to 30 hours. The product usually contained less than 0.1 per cent orthophosphate.

Standard reaction mixture and assay procedure. Unless otherwise stated, the following reaction mixture was used for assaying amino acid activation: Tris buffer, pH 7.4, 50 μmoles; $MgCl_2$, 5 μmoles; ATP, 2 μmoles; PP^{32}, 2 μmoles; amino acid mixture as indicated in the legend to Table II; NaF, 20 μmoles; hemolysate, 0.3 ml. The volume was made up to 1 ml with distilled water and the mixture was incubated for 20 minutes at 37° C. The reaction was stopped by the addition of 5 ml of 5 per cent trichloroacetic acid. The precipitated protein was removed by centrifugation, and 2 ml samples of the clear supernates were each treated with 1 ml of a 5 per cent suspension of acid-washed charcoal ("Norit A") in order to adsorb the ATP (8). The charcoal was washed 5 times with pH 4.5 acetate buffer containing 10 μmoles PP per ml, once with distilled water, and was then suspended in an alcohol: ether mixture (3: 1 vol/vol) and transferred quantitatively to planchets. The radioactivity was measured with an end-window Geiger-Mueller counter and the activity was calculated by dividing the total counts per minute incorporated into the ATP by the specific activity of the pyrophosphate added and expressed in terms of micromoles of PP exchanged per milligram protein of the homolysate or per 10^{11} RBC. Under the specified conditions the reaction closely followed a zero order course.

Analytical procedure. Total protein was determined by the method of Lowry, Rosebrough, Farr and Randall (9) and hemoglobin was estimated by the cyanmethemoglobin procedure (10). Inorganic phosphate was deter-

* This work was supported by a research grant from the Hadassah Medical Organization.

TABLE I

Summary of hematological data in the various groups of patients (range)

Clinical diagnosis	No. of patients	Hemoglobin	RBC*	Hematocrit	Reticulocytes	Serum iron†	Vitamin B_{12}‡
		gm %	*10^6/cu mm*	*%*	*%*	*µg %*	*µµg/ml serum*
Normal	30	13.4–15.2	4.6–5.2	44–49	0.1–0.6	79–108	180–420
Macrocytic§ hyperchromic anemia	20	6.5–9.2	1.5–3.2	21–36	1.2–3.1	67–110	60–190
Microcytic hypochromic anemia	16	4.5–7.2	2.7–3.9	19–34	0.8–2.8	0–38	200–450
Polycythemia vera	10	15.9–18.0	5.7–7.5	53–70	0.1–1.1	70–115	270–390

* RBC = red blood cells.
† Determined according to the method of Davies, Levin and Oberholzer (6).
‡ Determined according to the method of Grossowicz, Aronovitch and Rachmilewitz (7).
§ No patients with pernicious anemia (lack of intrinsic factor) or aplastic anemia have been included in this group.

mined by the method of Fiske and Subbarow (11). All the experiments were run in duplicate.

RESULTS

Conditions governing PP^{32}-ATP exchange in hemolysates. As shown in Table II, hemolysates prepared from normal RBC catalyzed the incorporation of P^{32}-labeled pyrophosphate into ATP.

Omission of amino acids from the otherwise complete reaction mixture resulted in a decrease in the rate of exchange to one-fourth to one-half of that observed with the complete system. It appeared likely that the considerable "background" activity obtained without the addition of amino acids was due to the appreciable amounts of amino acids released from the RBC in the course of lysis (12), especially since low substrate saturation levels are characteristic of the amino acid activating enzymes (2, 3). The validity of this assumption is strengthened by the finding that the ratio between

TABLE II

PP^{32}-ATP exchange in a normal blood hemolysate; requirements of the reaction *

	µmoles ATP^{32} / 100 mg protein	µmoles ATP^{32} / 10^{11} RBC†
"Complete amino acid mixture"‡	11.6	33.0
Amino acid mixture "A"‡	11.2	31.0
Tryptophan	8.7	25.3
No amino acids	5.1	13.7
No ATP	2.1	4.5
No Mg^{++}	3.8	6.3
P^{32} Pi§ added instead of PP^{32}	0	0
Pyrophosphatase added	0	0

* Conditions as described under Methods; see first section of text for abbreviations.
† Based on the red cell count performed immediately prior to lysis.
‡ "Complete amino acid mixture": arginine, aspartic, glycine, histidine, serine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, valine, glutamic, methionine, cysteine, tyrosine, proline. All amino acids were of the L-series and were added at 5×10^{-4} M final concentration. Amino acid mixture "A": arginine, glycine, histidine, serine, threonine, tryptophan, valine. Inorganic pyrophosphatase (40 µg) was added and the reaction mixture was pre-incubated for 5 to 10 minutes prior to the addition of ATP. (Kindly supplied by Dr. M. Kunitz, the Rockefeller Institute for Medical Research.)
§ Pi: inorganic phosphate.

TABLE III

Precipitation of the AAAE at pH 5.0 in the presence of sodium ribonucleate *

Treatment of hemolysate†		Complete amino acid mixture A	No amino acid B	Ratio A/B	No ATP
		% activity			*% activity*
None		100	33	3.0	10
pH 5.0‡		95	28	3.4	7
pH 5.0 + RNA§	Supernate	63	23	2.7	12
	Sediment	28	2.5	11.2	3

* The results are expressed in percentage of the activity obtained with untreated hemolysate, the latter set at 100 per cent. See footnotes * and ‡ to Table II for definitions.
† Hemolysate: particle-free supernate prepared from whole blood as described under Methods.
‡ The pH of the ice-chilled hemolysate was lowered to 5.0 by dropwise addition of 1 N acetic acid with continuous stirring. No precipitate was obtained. The pH of the hemolysate was immediately readjusted with 1 N Tris buffer to 7.2.
§ pH 5.0 + RNA: 30 mg/ml sodium ribonucleate [commercial yeast, RNA, purified according to the procedure of Woodward (13)] was added to the hemolysate with stirring until dissolved. Thereafter the solution was treated as above. The precipitate formed at pH 5.0 was sedimented by centrifugation and dissolved in a measured quantity of Tris buffer, 0.025 M, pH 7.2. The pH of the supernate was readjusted as above.

the endogenous and the amino acid-stimulated PP^{32}-ATP exchange activity increased from 1:3 in the crude hemolysate to 1:11 in a fraction of this hemolysate isolated by pH precipitation (Table III).

By examining various amino acids singly and in combination, it was found that amino acid mixture "A" (see legend to Table II) enhanced the exchange rate almost to the same extent as did a mixture of 18 natural amino acids, whereas 80 per cent of the maximal effect was elicited by tryptophan alone.

The radioactivity yielded without added ATP was about 15 per cent of that obtained with the complete reaction mixture. These counts could be almost entirely eliminated by using an isolated fraction of the hemolysate, indicating that this residual activity was due to the endogenous ATP content of the hemolysate (Table III).

Omission of Mg^{++} resulted in a roughly 70 per cent drop of the reaction rate (Table II).

P^{32}-orthophosphate failed to induce any detectable exchange with ATP when substituted for PP^{32} in the standard reaction mixture. The specificity of the system with respect to pyrophosphate was even more convincingly demonstrated by the finding that crystalline inorganic pyrophosphatase completely abolished the exchange reaction (Table II).

The pH of optimum PP^{32}-ATP exchange activity was found to range from 6.1 to 7.3. By varying the concentration of the amino acids in the reaction mixture the maximal rate of exchange was attained at levels close to 2×10^{-4} M.

The considerable adenosine triphosphatase and pyrophosphatase activity of the lysates, as measured by the release of orthophosphate from the respective substrates in the course of incubation, was entirely eliminated by the fluoride included in the reaction mixture. The stability of the assay system was also borne out by the fact that the rate of exchange remained practically constant during an experimental period which lasted for at least 40 minutes (Table IV).

Identification of the reaction product. At the end of the reaction the labeled ATP was adsorbed onto charcoal and the residual PP was removed by extensive washing. Thereafter the ATP was eluted with 50 per cent ethanol containing 0.5 M ammonia and isolated by paper electrophoresis, using 0.005 M citrate of pH 3.5 as the mobile phase. Under these conditions radioactivity was detectable only in the ATP spot located by its quenching of ultraviolet light. When the labeled ATP was hydrolyzed with 1 N HCl in a boiling water bath for 10 minutes, the radioactivity was no longer adsorbable with charcoal. The latter result indicated, therefore, that the P^{32}-label was entirely confined to the acid-labile terminal pyrophosphate moiety of ATP.

TABLE IV

Rate of PP^{32}-ATP exchange determined after increasing lengths of incubation in normal and polycythemic blood hemolysates *

Incubation time	Rate of exchange	
	Normal	Polycythemic
min	*mμmoles/hr/mg protein*	
10	35.4	24.9
20	35.4	21.6
30	33.0	
45	36.0	23.1

* Rate of exchange (R) was calculated according to the following equation (14):

$$R = \frac{(ATP) \times (PP^{32})}{(ATP) + (PP^{32})} \times \frac{2.3}{t} \times \log \frac{100}{100 - \% \text{ exchange}}.$$

Conditions as in Table II. Amino acid mixture "A" was used.

Fractionation. The AAAE of the rat liver cytoplasmic fraction are referred to by Hoagland, Keller and Zamecnik (15) as pH 5.0 enzymes because of their precipitating property at this pH as a nucleoprotein complex. The same procedure applied to whole blood hemolysates failed to produce a precipitate without affecting, however, the activity, provided that the pH was quickly readjusted to neutrality. It appeared likely that this difference in behavior is attributable to the virtual

TABLE V

Distribution of amino acid activating enzymes in various formed elements of the peripheral blood *

	μmoles ATP^{32} / 100 mg protein	μmoles ATP^{32} / 10^{11} cells	% of total† activity
Red blood cells	13.0	30.0	95.6
White blood cells	69.0	78.0	0.4
Platelets	30.0	37.0	4.0
Whole blood	13.57	31.3	100.0

* Conditions as described under Methods.
† The activity of the whole blood hemolysate was set at 100 per cent.

TABLE VI

Mean values of PP^{32}-ATP exchange in blood lysates from normal subjects and from anemic and polycythemic patients

	Normal				Macrocytic anemia				Microcytic anemia				Polycythemia			
	μmoles ATP^{32}		μmoles ATP^{32}		μmoles ATP^{32}		μmoles ATP^{32}		μmoles ATP^{32}		μmoles ATP^{32}		μmoles ATP^{32}		μmoles ATP^{32}	
	100 mg protein		10^{11} RBC		100 mg protein		10^{11} RBC		100 mg protein		10^{11} RBC		100 mg protein		10^{11} RBC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Amino acid mixture "A"*	10.6	3.0	31.0	6.8	17.8	4.7	42.3	6.6	24.4	5.3	50.0	14.5	6.9	1.1	15.3	3.2
Tryptophan	8.1	1.9	24.9	6.2	14.4	4.6	35.0	7.3	20.0	5.2	40.0	10.2	7.0	2.1	15.6	4.7
No amino acids	4.5	1.4	12.3	2.7	7.5	2.1	18.3	4.4	8.0	1.5	14.1	4.1	2.9	0.4	6.7	1.3
No ATP	2.0	0.8	5.0	2.0	2.1	0.5	6.0	1.5	1.9	0.6	3.5	1.5	1.2	0.2	2.7	0.4

* Amino acid mixture "A"; see footnote‡ to Table II. Conditions otherwise as in Table II.

absence of nucleic acids in the hemolysate. This seems to be confirmed by the finding that when RNA was added to the hemolysate, a precipitate was obtained at pH 5.0 which contained about 25 per cent of the total AAAE activity present in the original hemolysate (Table III). No further fractionation was attempted.

The distribution of AAAE in the peripheral blood cells. The distribution of the AAAE in the various formed elements of the peripheral blood was studied by determining the PP^{32}-ATP exchange in lysates prepared separately from erythrocytes, leukocytes and platelets isolated by a modification of a conventional technique (16). As evident from Table V, the bulk of the total blood cell activity resides in the RBC. On the other hand, the specific activity of the lysates calculated per unit number of cells is highest in the leukocytes and lowest in the RBC.

Leukocytes and RBC injured by a single hypotonic treatment retained about as much activity as that released into the supernatant solution. The residual activity could be solubilized by subjecting the ghosts resedimented by centrifugation to repeated osmotic lysis or to sonication for 2 to 3 minutes. On the other hand, ghosts of platelets obtained after a single hypotonic lysis exhibited no detectable PP^{32}-ATP exchange. It may be pointed out in this connection that no PP^{32}-ATP exchange could be demonstrated with any of the

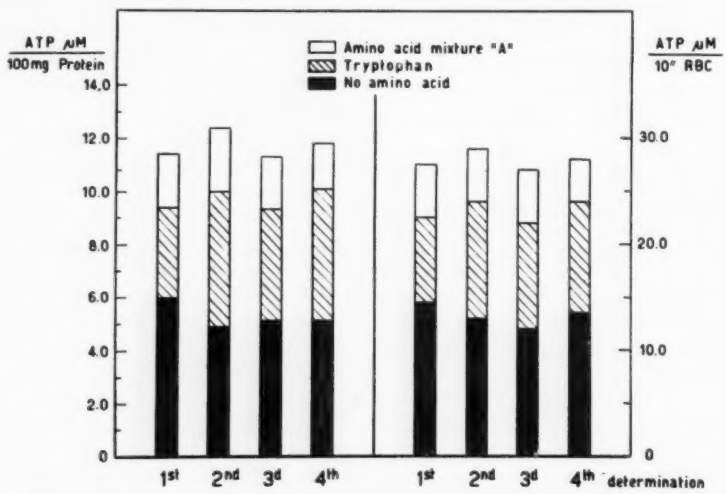

FIG. 1. REPEATED DETERMINATIONS OF PP^{32}-ATP EXCHANGE AT BIWEEKLY INTERVALS.

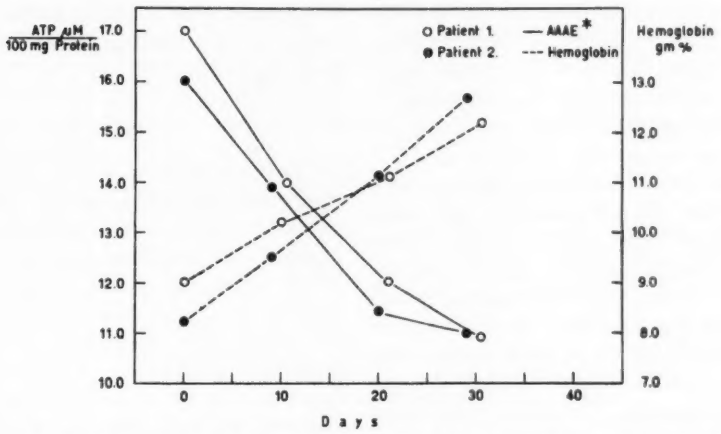

FIG. 2. GRADUAL DECREASE OF PP^{32}-ATP EXCHANGE PARALLELING THE INCREASE IN HEMOGLOBIN VALUES IN TWO ANEMIC PATIENTS. * Amino acid activating enzyme.

intact cells of the peripheral blood, apparently owing to their impermeability to ATP.

Amino acid activation capacity of normal blood hemolysates. Table VI summarizes the results of a series of determinations of PP^{32}-ATP exchange performed on blood cell hemolysates from 30 normal individuals. There was a considerable variation in the values obtained in different persons. However, when repeated determinations were made on the same subject at definite time intervals, the levels of activity were found to be remarkably constant (Figure 1).

Macrocytic and microcytic anemias. Patients with macrocytic anemia were selected from a large group of women diagnosed as anemic during pregnancy and postpartum, while cases with uncomplicated hypochromic iron deficiency anemia were chosen from subjects available at the Hematology Outpatient Department of the Hadassah University Hospital. In both groups the amino acid-dependent pyrophosphate exchange was markedly higher than the average values observed in the normal group, the increase being particularly striking in the hypochromic anemia patients (Table VI). The significance of this correlation between the biochemical alteration and the patho-

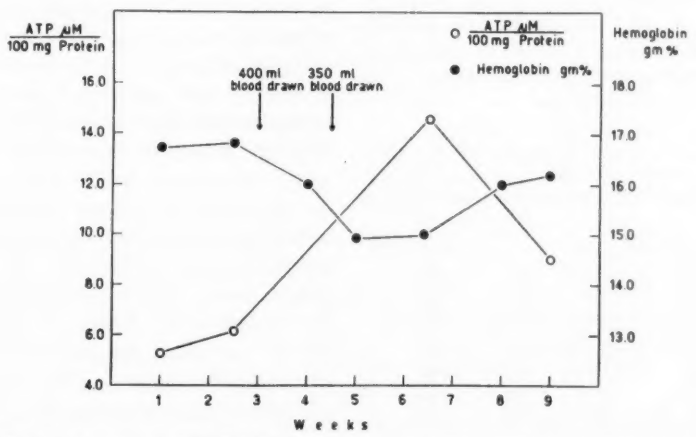

FIG. 3. INCREASE OF PP^{32}-ATP EXCHANGE FOLLOWING REPEATED VENISECTIONS IN A POLYCYTHEMIC PATIENT.

logical condition is further borne out by the follow-up of six treated patients in whom progressive decline of activity down to the normal range paralleled the improvement in the hematological status. Two representative cases are illustrated in Figure 2.

Polycythemia vera. In untreated patients with polycythemia vera the pyrophosphate exchange was of a considerably lower order than in normal subjects. Addition of tryptophan alone to the reaction mixture elicited maximal response which was not increased by further addition of other amino acids (Table VI). In these patients the activity rose substantially after repeated venesections, gradually returning, however, to the original low level within several weeks following the last phlebotomy (Figure 3).

Osmotic fragility and AAAE. The high activity found in anemic patients suggested the possibility that the elevated values were due to a "shift to the left" in the age of the red cell population in view of the shortened RBC life span characterizing these conditions (17, 18). This possibility was explored by examining the enzymic activity as a function of the osmotic fragility which is known to vary directly with the age of the red cells (19).

Table VII shows that the activity per unit number of red cells lysed remained roughly constant. The anemic cells showed an essentially similar behavior. It may be relevant to mention in this context that there was no correlation between the degree of reticulocytosis and the PP^{32}-ATP exchange activity in the various groups studied. Thus the AAAE exhibits a uniform pattern of distribution in the RBC population, differing in this respect from the behavior of some other enzymes, as described by Marks, Johnson, Hirschberg and Banks (20).

TABLE VII
PP^{32}-ATP exchange in relation to osmotic fragility

% NaCl solution	Normal		Microcytic hypochromic anemia	
	% Activity in supernate	% Hemoglobin in supernate	% Activity in supernate	% Hemoglobin in supernate
0.4	30	39	14	17
0.35	90	92	60	51
0.30	95	93	90	84
	100*	100	100	100

* The values obtained after lysis with distilled water are arbitrarily considered as 100 per cent.

DISCUSSION

The occurrence of amino acid activating enzymes in mature red blood cells which are known to be incapable of protein production is surprising in view of the presumed essential role of these enzymes in the biosynthesis of protein (5). It is possible, of course, that the AAAE perform in the mature RBC a different function which remains to be elucidated. It is likewise conceivable, however, that the system concerned represents a nonfunctional rudiment carried over from the nucleated stages of erythropoiesis.

The pronounced differences in the AAAE activity observed between blood cells from normal subjects and those from patients with various hematological disorders seem to reflect early disturbances occurring at some stage of the RBC maturation rather than a change in the type of the mature blood cells. The uniform distribution of this activity within the blood cell population, regardless of the differences in the osmotic fragility in both normal subjects and anemic patients, indicates a lack of correlation between the level of AAAE and the age of RBC. This conclusion is also in line with the observation that the reticulocyte count did not seem to affect significantly the activity observed within a given group.

The nature of the biochemical lesion responsible for the observed deviations from the normal level of activity is not clear. It is tempting to speculate that the enhanced AAAE activity found in the anemic disorders described manifests an attempt of the hematopoietic system to compensate for the deficient hemoglobin synthesis. This hypothesis would also account for the gradual decrease of the activity, down to normal values, occurring some time after a normal hemoglobin level had been attained. On the other hand, the decrease in the AAAE level observed in patients with polycythemia vera may reflect an opposite tendency, aimed to restrain the excessive activity of the erythropoietic tissue. It is interesting to mention in this connection that the AAAE activity determined in the RBC of a limited number of patients with secondary polycythemia was found to

be within the normal range. As to the transient increase of the PP^{32}-ATP exchange observed in polycythemic patients following repeated venesections, this represents a response of the protein synthesizing system to the drastic reduction in the circulating red cell mass.

Thus the mechanisms discussed above can be viewed as two facets of a homeostatic device operating through a restrictive effect exerted by the red cell proteins on the formation of the enzymic system mediating their own biosynthesis. No information is available as to the nature of the regulatory factor(s) involved in this mechanism.

Further work is required to test the validity of this interpretation.

SUMMARY

Human whole blood hemolysates were found to catalyze an amino acid-dependent exchange of pyrophosphate with adenosine triphosphate. Out of a number of amino acids tested, tryptophan showed highest activity, amounting to about 80 per cent of the total exchange rate elicited by a mixture of 18 natural amino acids. Magnesium ions proved to be an essential component of the reaction.

Other factors influencing the reaction rate, such as amino acid concentration and pH optimum, were determined. The product of the exchange reaction was characterized as adenosine triphosphate labeled in the two terminal phosphate groups.

The bulk of the total amino acid activating enzyme content of the hemolysates was present in the red blood cells, while leukocytes showed the highest specific activity. The distribution of these enzymes in the red blood cell population showed no correlation with its osmotic fragility pattern.

The level of amino acid activating enzyme in erythrocytes derived from patients with macrocytic and microcytic anemias was markedly increased as compared with normal values. On the other hand, a significant decrease of this activity was observed in red cells obtained from patients with polycythemia vera. These elevations above the normal range tended to regress parallel with hematological improvement.

The significance of these enzymes in mature red cells and the possible mechanism underlying the deviations from the normal level in various hematologic disorders are discussed.

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OBSERVATIONS ON THE FUNCTIONAL HOMOGENEITY OF THE NEPHRON POPULATION IN THE CHRONICALLY DISEASED KIDNEY OF THE DOG *

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Most forms of chronic renal disease result in extensive structural alterations of the renal parenchyma. Both glomeruli and tubules may exhibit a spectrum of anatomic abnormalities which give the constituent nephrons an appearance of extraordinary heterogenity. The degree to which these diversified structural changes alter the function of the surviving nephrons is of central importance to the clarification of the functional organization of the diseased kidney. In the present studies an attempt has been made to evaluate the functional homogeneity of the chronically diseased kidney in the dog, using the glucose titration technique (1, 2). Three forms of experimental renal disease have been employed. In each, anatomic heterogeneity of the renal parenchyma was a striking and consistent feature. In order to compare the function of the diseased organs with kidneys uninvolved by disease, the lesions were induced in one kidney only, and the contralateral kidney served as a control in each animal.

METHODS

Twelve glucose titration experiments were performed on 9 young, adult, trained, female mongrel dogs with unilateral renal disease. Four animals had unilateral pyelonephritis (3), three had unilateral aminonucleoside nephritis (4) and two had unilateral antikidney serum nephritis (5).

The animals were studied in the fasting and unanesthetized state and in the standing position. Thirty ml per kg of tap water was administered by nasogastric tube in advance of the experiment in order to insure adequate urine flows during the initial urine collection periods. Glucose was infused intravenously in 0.45 per cent saline at the rate of 5.0 ml per minute throughout the experiment. Four levels of glucose infusion were employed. Three per cent glucose was infused during a 30 to 40 minute equilibration period and the first 3 clearance periods. Eight per cent glucose was then infused during a 5 to 15 minute equilibration period and the 3 succeeding clearance periods. Thereafter, 11 per cent glucose was infused and 3 urine collections were made without an intervening period of equilibration. Finally 20 per cent glucose was infused, a 30 minute equilibration period was allowed to assure plasma glucose levels well in excess of the tubular reabsorptive maximum and the last 3 clearance periods were obtained. Urine from the diseased and contralateral control kidneys was collected simultaneously from permanent cystotomy tubes draining individual hemibladders. Continuous arterial blood samples were collected throughout each urine collection period by means of an indwelling femoral arterial polyethylene catheter.

Glomerular filtration rate was estimated by the exogenous creatinine clearance. Creatinine was measured according to the method of Bonsnes and Taussky (6); and over the range of glucose concentrations observed in plasma and urine, no interference with creatinine analysis occurred. Glucose was determined using a specific enzymatic (i.e., glucose oxidase) technique (7). Additional details of the experimental procedures employed have been described previously (4).

RESULTS

In all dogs the diseased kidney was contracted and histologic examination revealed marked structural abnormalities, the details of which varied with the type of underlying renal disease. The values for glomerular filtration rate (GFR) and maximal rate of glucose reabsorption ($Tm_{glucose}$) were consistently decreased for the diseased kidneys relative to the contralateral control kidneys. Values for GFR ranged from 3.6 to 21 ml per minute (mean, 12) for the diseased, and from 22 to

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TABLE I

Glomerular filtration rate (GFR), maximal rate of glucose reabsorption ($Tm_{glucose}$) and $GFR/Tm_{glucose}$ ratios for diseased and contralateral control kidneys

Dog	Lesion*	Weight	GFR		$Tm_{glucose}$		$GFR/Tm_{glucose}$	
			Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
		kg	*ml/min*		*mg/min*			
1	Pyelo.	9.5	10	23	44	87	0.23	0.26
			11	22	42	81	0.26	0.27
2	Pyelo.	10.5	16	29	52	90	0.31	0.32
			15	23	51	78	0.30	0.30
3	Pyelo.	10.5	3.6	46	14	176	0.26	0.26
4	Pyelo.	15.0	24	51	65	135	0.38	0.38
5	A.N.	8.0	6.2	37	15	91	0.39	0.40
			4.8	30	12	77	0.39	0.39
6	A.N.	15.2	16	50	57	186	0.28	0.27
7	A.N.	15.4	18	45	49	113	0.37	0.39
8	A.K.S.	13.7	22	42	69	148	0.32	0.28
9	A.K.S.	15.9	4.6	44	22	202	0.21	0.22

* Exp. and Cont. refer to experimental (i.e., diseased) and control kidneys. Pyelonephritis is designated as Pyelo., aminonucleoside nephritis as A.N., and antikidney serum nephritis as A.K.S.

51 ml per minute (mean, 37) for the control organs. Values for $Tm_{glucose}$ ranged from 12 to 69 mg per minute (mean, 29) for the diseased kidneys, and from 77 to 202 mg per minute (mean, 119) for the control organs. Of considerable interest is the fact that in all dogs, the ratios of $GFR/Tm_{glucose}$ for the separate kidneys were quite comparable. Values for GFR, $Tm_{glucose}$ and $GFR/Tm_{glucose}$ are shown in Table I.

In Table II, a representative glucose titration experiment is shown for an animal with unilateral renal disease. Plasma glucose concentrations were increased from an initial fasting level of 80 to 714 mg per 100 ml. Glucose reabsorption was essentially complete bilaterally until the plasma glucose concentration exceeded 250 mg per 100 ml. Some degree of glycosuria occurred bilaterally before the load of glucose equaled the Tm values (Period 5), and a limited splay in the respective titration curves resulted. This was slightly more marked for the diseased than for the control kidney in this experiment (Period 6);

TABLE II

A glucose titration experiment in a representative dog with unilateral renal disease *

Period	Time	Plasma glucose	Exp. kidney					Control kidney				
			GFR	Glucose excretion	$T_{glucose}$	$\frac{T}{Tm}$	$\frac{Load}{Tm}$	GFR	Glucose excretion	$T_{glucose}$	$\frac{T}{Tm}$	$\frac{Load}{Tm}$
	min	*mg/100 ml*	*ml/min*	*mg/min*	*mg/min*			*ml/min*	*mg/min*	*mg/min*		
						3% glucose						
1	0–16	123	14.5	0	17.8	0.36	0.36	21.7	0	26.6	0.34	0.34
2	16–30	136	15.9	0	21.6	0.43	0.43	23.2	0	31.6	0.41	0.41
3	30–40	144	15.2	0	21.8	0.43	0.43	23.6	0	33.9	0.44	0.44
						8% glucose						
4	49–62	252	14.6	0.3	36.4	0.72	0.73	23.8	0.4	59.5	0.77	0.77
5	62–75	298	15.3	2.4	43.2	0.86	0.91	23.6	3.8	66.5	0.85	0.91
6	75–89	350	15.1	5.4	47.4	0.94	1.04	24.7	8.9	77.6	1.00	1.11
						11% glucose						
7	89–103	405	15.8	10.4	53.6	1.07	1.27	25.7	17.8	86.3	1.11	1.34
8	103–119	490	14.6	20.5	51.0	1.02	1.42	22.1	33.4	74.9	0.96	1.39
9	119–129	500	14.5	26.6	45.9	0.91	1.44	23.5	42.0	75.5	0.97	1.51
						20% glucose						
10	142–150	664	15.7	51.7	52.5	1.05	2.08	22.7	76.2	74.5	0.96	1.94
11	150–159	714	15.6	60.2	51.2	1.02	2.22	23.2	87.6	78.0	1.00	2.13

* $T_{glucose}$ refers to glucose reabsorption. Load refers to the filtered load of glucose and is equal to the product of GFR × plasma glucose concentration. The value for Tm for both kidneys was calculated as the mean value for $T_{glucose}$ for Periods 6 through 11. $Tm_{glucose}$ was 50.3 mg/min for the experimental kidney and 77.8 mg/min for the control kidney.

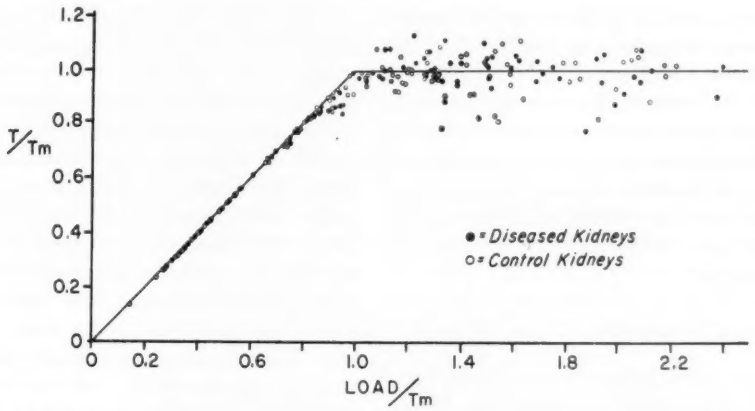

FIG. 1. A MASS PLOT OF THE DATA OBTAINED IN 12 ANIMALS UNDERGOING GLUCOSE TITRATION EXPERIMENTS. T/Tm refers to the observed rate of glucose reabsorption expressed as a fraction of the Tm for the same kidney. Load/Tm refers to the filtered load expressed as a fraction of the Tm.

however, this relationship was not a consistent one.

In Figure 1, a mass plot is shown of the data obtained in 12 experiments; 120 observations for the diseased kidneys are compared with the same number of observations, simultaneously obtained, for the control kidneys. Gross inspection of the data suggests that there is no greater titration splay for the diseased than for the normal organs.

Figure 2 depicts the mean smoothed titration

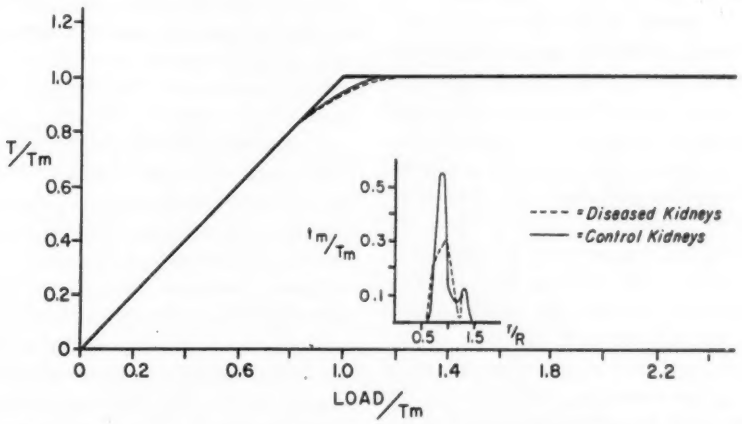

FIG. 2. THE MEAN TITRATION CURVES FOR THE GROUP DATA PRESENTED IN FIGURE 1. The frequency distribution curves for the two sets of group data are shown in the insert. The symbols, tm/Tm and r/R are those conventionally used for this plot: tm/Tm represents the rate of glucose reabsorption in saturated nephrons expressed as a fraction of the whole kidney Tm, and thus this term serves as an index of the fraction of the total nephron population; r/R provides a ratio of the glomerular tubular balance (with respect to glucose) for individual nephrons, or categories of nephrons, to the mean value for glomerular tubular balance for the whole kidney. A detailed discussion of the methods of calculating both the mean titration curves and the frequency distribution curves is contained in the paper by Smith and co-workers (2).

curves obtained according to the method of Smith, Goldring, Chasis, Ranges and Bradley (*2*) for the group data shown in Figure 1. The shape of the curve for the diseased kidneys is essentially indistinguishable from that for the normal kidneys. Frequency distribution curves for the group data are shown in the insert in Figure 2. Statistical analysis of the two distribution curves shows no significant difference between either the mean values or the variances.

DISCUSSION

The glucose titration technique provides a physiologic means of exploring the functional homogeneity of the constituent nephrons of a given kidney. The rationale depends upon the assumptions, that for each functioning nephron: *1*) the proximal tubule has a maximal rate at which it may transfer glucose (i.e., a Tm); *2*) reabsorption will be essentially complete until the Tm is achieved; and *3*) the rate of glucose reabsorption will remain constant after the load exceeds the Tm and all filtered glucose in excess of the reabsorbed moiety will be excreted.

The titration is performed by increasing the concentration of glucose in the plasma progressively from normal levels to values sufficiently high to produce marked and sustained glycosuria. At any point in time, the load of glucose presented to the reabsorbing sites in each individual nephron represents the product of the volume of glomerular filtrate entering the unit times the concentration of glucose in the filtrate. Because all of the nephrons of a kidney are perfused by plasma of identical composition, the glucose concentration may be presumed to be the same for all functioning units. Hence any difference in the time of appearance of glycosuria between adjacent nephrons should reflect a corresponding difference in the balance between GFR and proximal glucose reabsorptive capacity. When applied to a whole kidney, therefore, the technique provides an index of the consistency of glomerulotubular balance (with respect to glucose) for the constituent nephrons.

If there is a uniform (i.e., homogeneous) relationship between filtration and glucose reabsorption, all nephrons should reach their Tm at the same plasma glucose concentration. Hence the filtered glucose should be completely reabsorbed until the plasma concentration exceeds a critical level; and thereafter the rate of glucose reabsorption should remain constant. However, if there is functional heterogeneity, the pattern should be different. Any group of nephrons in which the volume of filtrate is disproportionately large with respect to glucose reabsorptive capacity should reach Tm before the remaining units, and glucose should be delivered into the urine at a relatively low plasma glucose concentration. Alternatively, nephrons in which the volume of filtrate is disproportionately small relative to reabsorptive capacity should continue to reabsorb filtered glucose until plasma glucose concentrations become very high and after the remaining units have reached their Tm.

Recently, Bradley, Laragh, Wheeler, MacDowell and Oliver (8) have reported observations which lend considerable authority to the glucose titration technique as a means of evaluating nephron homogeneity. These workers have found that in the dog, the relationship between glomerular and proximal tubular function as assessed by glucose titration curves correlates precisely with anatomic measurements of glomeruli and their attached tubules (i.e., glomerular area versus proximal tubular volume) in nephrons from the same kidneys which have been isolated by microdissection.

In the normal dog kidney, Shannon, Farber and Troast (9) and Kessler, Heidenreich and Pitts (10) have found that glucose reabsorption generally is essentially complete until the Tm is reached, following which reabsorption remains relatively constant despite increasing levels of plasma glucose. The fact that only a limited titration splay exists suggests that the nephron population of the normal dog kidney is basically a homogeneous one. The glucose titration technique would therefore appear to be well suited for the evaluation of the homogeneity of the nephron population of the diseased as well as the normal kidney. If anatomic heterogeneity results in corresponding functional heterogeneity, the diseased kidney should exhibit a splay in its titration curve considerably greater than that of a normal kidney.

In the present studies, glucose titration curves for the diseased kidneys of dogs were compared with the ideal curve for a homogeneous nephron

population and also with the curves obtained simultaneously for contralateral kidneys which were free of disease.

Analysis of the data for individual animals revealed a high degree of comparability in titration curves between diseased and contralateral kidneys. In most instances the titration splay was minimal for both organs, and the magnitude of the splay typically was no greater for the diseased than for the contralateral kidneys. In a composite plot of group data (Figure 1), the titration splay was small for both diseased and intact organs; and the mean titration curves for the two sets of data were virtually superimposable. Comparison of the two frequency distribution curves showed no significant differences between the means or the variances. Statistically, therefore, the nephrons of the diseased kidneys could not be differentiated from those of the intact organs on the basis of their respective patterns of glucose transport.

Within the limits of accuracy of the glucose titration technique, the present observations are consistent with the thesis that the relationship between the volume of glomerular filtrate and the glucose reabsorptive capacity of the attached proximal tubules remains uniform in the surviving nephrons of the experimentally diseased kidney in the dog. At least two explanations may be considered for this phenomenon. The first is that the nephrons in a diseased kidney adhere functionally to what is tantamount to an all or none pattern. This would require that a nephron which sustains sufficient structural damage to either its glomerulus or proximal tubule to modify ultrafiltration or glucose transport would become functionless. The loss of nephron units could be either temporary or permanent depending on the nature of the pathologic process.

The second theoretic explanation for the observed functional homogeneity does not require an all or none hypothesis, but introduces certain other conceptual difficulties. This would hold that a decrease in the volume of glomerular filtrate induced by anatomic alterations in a filtering glomerulus would be accompanied by a proportional decrease in $Tm_{glucose}$ for the attached proximal tubule, such that the balance between filtration and glucose reabsorption would remain comparable with that for uninvolved nephrons as well as for other damaged but still functioning nephrons. Because the filtration fractions remain quite comparable between diseased and intact kidneys of individual animals, it is theoretically possible that a preferential decrease in GFR in an involved group of nephrons would be accompanied by a proportional decrease in postglomerular blood flow; and one possible means of autoregulation of glomerulotubular balance (with respect to glucose reabsorption) would involve a modulation of the rate of glucose transport by some function of the peritubular capillary blood flow. However, if a mechanism for autoregulation of glomerulotubular balance does exist in these diseased kidneys, it must also be capable of acting in a reverse fashion. Thus a primary decrease in glucose Tm due to an intrinsic structural abnormality in a proximal tubule would have to initiate a proportional decrease in the volume of fluid filtered by an appended glomerulus. In addition, the concept of autoregulation in damaged nephrons must explain the fact that some other tubular functions (e.g., Tm_{PAH}, $Tm_{phosphate}$, ammonia and titratable acid excretion) retain the same relationship to GFR in experimentally diseased kidneys as in contralateral control organs.

SUMMARY

Glucose reabsorption has been measured during rising plasma glucose concentrations in dogs with one of three types of chronic unilateral renal disease. The glucose titration curves for the diseased kidneys were compared with those simultaneously obtained for the contralateral intact organs. Both in studies on individual animals and in a composite plot of group data for 12 experiments on 9 dogs, the patterns of diseased and contralateral kidneys were quite similar. Analysis of the group data demonstrated no appreciable titration splay for either diseased or normal kidneys; and no significant differences were found between the frequency distribution curves for the two sets of data. On the basis of these observations, it has been suggested that the balance between the volume of glomerular filtrate and the glucose reabsorptive capacity of the attached proximal tubules remained uniform for the surviving nephrons of the diseased organs, despite evidence of marked anatomic heterogeneity. Within the limitations of the glucose titration method, the residual functioning nephrons of the experimentally diseased

kidney in the dog would therefore appear to constitute a basically homogeneous population.

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IDIOPATHIC HYPERLIPEMIA: METABOLIC STUDIES IN AN AFFECTED FAMILY *

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The clinical syndrome of idiopathic hyperlipemia is characterized by lactescent blood plasma in the postabsorptive state. The milky appearance of the plasma is caused by its abnormally high content of triglyceride-rich lipoproteins of high molecular weight. Omission of fat from the diet of such patients decreases the concentration of triglycerides, suggesting that defective removal of exogenous fat from the circulation may be an important metabolic abnormality in this syndrome.

The heparin-activated enzyme, lipoprotein lipase, is thought to be concerned with removal of newly absorbed fat from the blood. The basis for this assumption rests on the accelerated removal of chylomicrons from the blood after injection of heparin, which liberates the enzyme into the circulation, resulting in lipolysis in the blood plasma itself (1); the retarded removal of chylomicrons from the blood after administration of antiheparin agents such as protamine sulfate (2); the preferential hydrolysis by the enzyme of triglycerides associated with lipoproteins (3); and the rapid conversion of the fatty acids of intravenously injected chylomicrons into circulating free fatty acids (4). A deficiency of lipoprotein lipase would be expected to result in excessive and prolonged chylomicronemia after ingestion of fat, and thus might be a basic cause of idiopathic hyperlipemia.

We undertook the present investigation to examine this hypothesis and to study lipid transport in the blood of three hyperlipemic siblings of a family of eight, who were previously described by Gaskins, Scott and Kessler (5). The results suggest that a deficiency of lipoprotein lipase is the cause of the hyperlipemic state in the affected siblings.

* Portions of this work were presented to the American Society for the Study of Arteriosclerosis (Circulation 1955, **12**, 485) and at the Third International Conference on Biochemical Problems of Lipids, Brussels, July 1956 (The Blood Lipids and the Clearing Factor; Lederberg/Ghent, Erasmus, 1956, pp. 265–273).

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METHODS

Subjects. The subjects, who were members of one family, were W.P (father), D.P. (mother), F.P. (daughter), C.P. and A.P. (normal sons) and L.P., J.P. and P.P. (hyperlipemic sons). For purposes of comparison two unrelated men with idiopathic hyperlipemia were also studied. (For clinical data, see the Appendix.) Physicians and healthy volunteers hospitalized at the Clinical Center served as control subjects.

Procedures. The five hyperlipemic subjects, all males, were hospitalized in the Clinical Center and given measured diets designed to maintain their weight and containing 1 g of fat per kg of body weight per day. The diet was altered by varying the carbohydrate and fat content; calories were kept constant at the initial level unless otherwise noted. For studies of the effects of a single load of fat, the subjects were given a standard meal containing 1.5 g of fat per kg of body weight in the form of bacon, eggs and oleomargarine, but practically no carbohydrate (6).

Venous blood samples were taken routinely about 15 hours after the last meal. Serum was obtained from blood clotted at room temperature for lipid and lipoprotein analyses. Samples for estimation of plasma free fatty acids were mixed with solid sodium oxalate, 1.5 mg per ml of blood, and placed immediately in ice water. Samples taken after injection of heparin [1] (post-heparin samples) were centrifuged rapidly at 2° C and the plasma was extracted immediately thereafter. Blood samples for estimation of lipoprotein lipase activity were mixed with disodium ethylenediamine tetraacetate, 1 mg per ml of blood, and placed immediately in ice water.

Analytical methods. Blood serum was extracted in ethanol: acetone (1:1) or chloroform: methanol (2:1) and analyzed for total and free cholesterol (7), lipid phosphorus (8) and total lipids (9). Phospholipids were estimated as lipid phosphorus × 25, and triglycerides were calculated by difference (6). Lipoproteins were separated into three fractions at densities 1.019 (very low density, including chylomicrons), 1.063 (low density) and 1.21 (high density) in the preparative ultracentrifuge and analyzed as described previously (10). Free fatty acids were determined as described in previous publications (11, 12). Glycerol was measured by a modifica-

[1] Heparin sodium, 1,000 USP units per ml, Upjohn Co.

TABLE I

Serum lipoprotein fractions in P. family

Fraction	Total cholesterol	Phospholipids	Protein
	mg/100 ml	*mg/100 ml*	*mg/100 ml*
Very low density (< 1.019)			
L.P. (hyperlipemic brothers)	216	220	79
J.P. (hyperlipemic brothers)	341	298	75
P.P. (hyperlipemic brothers)	401	416	93
D.P. (mother)	17	24	17
W.P. (father)	60	64	39
Healthy young men	26 (14–36)	31 (15–42)	~12
Low density (1.019–1.063)			
L.P.	9	19	30
J.P.	14	20	27
P.P.	5	13	30
D.P.	198	130	101
W.P.	162	108	105
Healthy young men	97 (61–133)	69 (45–79)	~62
High density (1.063–1.21)			
L.P.	4	36	81
J.P.	9	25	65
P.P.	5	25	69
D.P.	57	92	170
W.P.	30	68	134
Healthy young men	43 (31–48)	~84	~150
Residual (> 1.21)			
L.P.	0	18	
J.P.	0	25	
P.P.	0	17	
D.P.	0	22	
W.P.	0	27	
Healthy young men	0	~27	

tion of the method of Lambert and Neish (13).[2] Unless otherwise noted, optical clearing of plasma was measured as follows: reagents and plasma were warmed to 37° C; 0.1 ml of 0.5 per cent coconut oil emulsion (made by diluting Ediol, Schenley Laboratories), 0.1 ml of 0.5 per cent disodium ethylenediamine tetraacetate, 0.3 ml of saline-phosphate buffer (pH 7.4, ionic strength 0.1), and 0.5 ml of plasma were mixed in a colorimeter tube and placed in a water bath at 37° C. Serial readings of optical density were made in a Coleman, Jr. spectrophotometer at 500 mμ. Respiratory quotient was measured after subjects had fasted overnight.

RESULTS

Nature of the lipemia

The total lipid concentration of serum from the hyperlipemic siblings when on their usual diet was 3,000 to 5,000 mg per 100 ml. When the serum was kept at 3° C for a few days, the turbid material separated as a creamy layer, leaving an optically clear subnatant fluid which contained only 100 to 200 mg of lipid per 100 ml. Table I shows the distribution of certain constituents of lipoprotein fractions separated from these sera and from sera obtained from the subjects' parents and from healthy 20 to 30 year old men (10). In Table II the composition of the very low density lipoprotein fraction (14) in the sera of the three brothers and of the two unrelated men with idiopathic hyperlipemia is compared with that of chylomicrons isolated by flotation from pooled serum obtained from the American Red Cross Blood Donor Service. The concentration of very low density lipoproteins in the blood of the hyper-

[2] We are indebted to Dr. Edward Korn for performing these analyses.

TABLE II

Composition of very low density lipoproteins in sera from individuals with idiopathic hyperlipemia

Subject	Component: % by weight				
	Cholesterol esters	Free cholesterol	Phospholipids	Triglycerides	Protein
L.P.	5.3	2.9	6.2	83.4	2.2
J.P.	6.4	3.4	7.0	81.6	1.6
P.P.	6.9	3.3	6.6	81.5	1.7
F.M.	7.9	4.3	12.1	73.5	2.2
L.W.	13.0	4.3	13.6	64.0	5.1
Pooled serum chylomicrons	6.1	3.1	7.1	81.3	2.5

lipemic brothers was markedly increased. The concentration of the low density and high density fractions was considerably reduced, compared with that in normal men, and their content of cholesterol was disproportionately low (Table I). In one of the two unrelated subjects the percentage of cholesterol esters in the very low density lipoproteins was considerably greater than that of chylomicrons; in both, the concentration of phospholipids was greater, while the proportion of triglycerides was less (Table II).

Effects of diet

Since the results of dietary changes were similar in the three brothers, detailed results of studies on L.P. only are presented (Figure 1). With the initial diet, which contained 1 g of fat per kg body weight [3] per day, serum triglyceride concentration fell rapidly to about 1,300 mg per 100 ml. After 21 days the dietary fat was reduced by 90 per cent. Serum triglyceride concentration fell rapidly to about 250 mg per 100 ml, and the serum became optically clear on Day 24. No significant change was produced by cutting caloric intake in half for five days (Days 28 to 33). Respiratory quotient during this period was 0.73. When the diet containing 1 g of fat per kg per day was resumed (Day 39), triglyceride concentration rose slightly above the previous value. Later (Day 61) fat intake was increased to 3 g per kg per day, and triglyceride concentration rose strikingly to 5,000 mg per 100 ml. The concentration fell dramatically again when the dietary fat content was reduced to 0.5 g per kg (Day 67). Changes in the total and free cholesterol and phospholipid concentrations of the serum paralleled the changes in triglycerides but were less pronounced.

Data on the serum lipoprotein fractions during this experiment are shown in Figure 2. In the very low density lipoprotein-chylomicron fraction the concentrations of total cholesterol, phospholipids and protein followed closely the rise and fall of serum triglycerides. However, their concentrations remained considerably above the normal values shown in Table I. In the low density lipoproteins the changes were in the opposite direction. Alterations in the high density lipoproteins were variable. Total cholesterol concentration changed relatively little; variations in phospholipid concentration could not be correlated definitely with alterations in diet. The concentration of phospholipids in the residual serum proteins was unaltered throughout the study. A comparative study was made on one of the unrelated subjects with

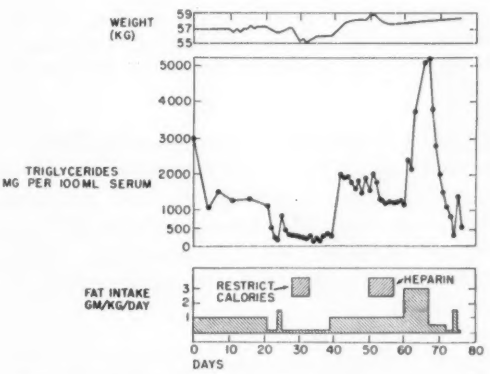

FIG. 1. EFFECTS OF ALTERATIONS IN DIET AND ADMINISTRATION OF HEPARIN, 200 MG SUBCUTANEOUSLY EVERY 12 TO 24 HOURS, ON SERUM TRIGLYCERIDE CONCENTRATION IN HYPERLIPEMIC SUBJECT L.P. A single fat-rich meal was fed on Days 23 and 74.

[3] Hereafter written as g per kg (of body weight understood).

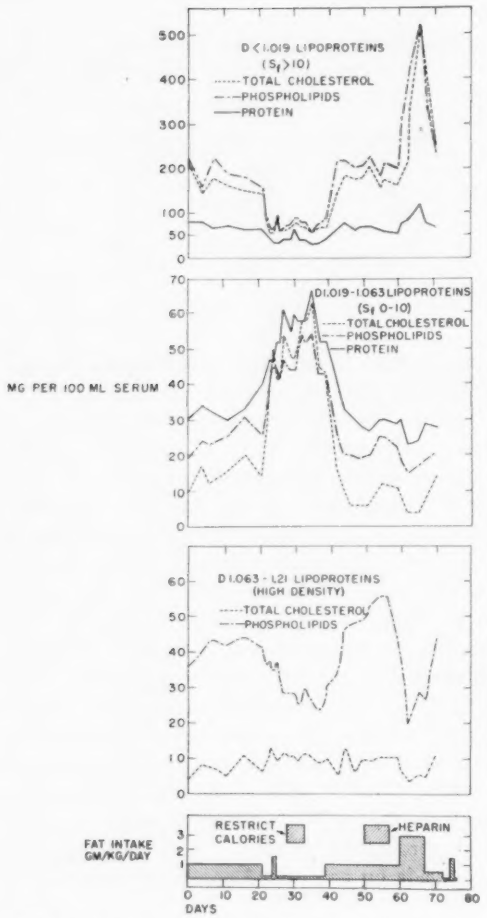

FIG. 2. EFFECTS OF ALTERATIONS IN DIET AND ADMINISTRATION OF HEPARIN ON CHEMICAL CONSTITUENTS OF THREE SERUM LIPOPROTEIN CLASSES IN HYPERLIPEMIC SUBJECT L.P.

idiopathic hyperlipemia. As shown in Figure 3, reduction of fat intake was associated with a fall in triglyceride concentration and reciprocal changes in cholesterol concentration in the very low and low density lipoprotein fractions. Serum cholesterol concentration remained practically unchanged.

Effects of single loads of fat

After a period of stabilization on the diet containing 0.1 g of fat per kg per day, the subjects were fed single meals containing 1.5 g of fat per kg. The results of a representative study are

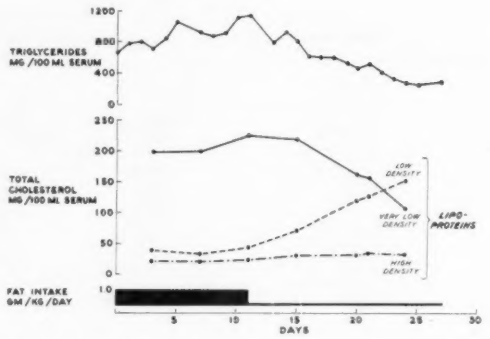

FIG. 3. EFFECT OF ALTERATIONS IN DIET ON TRIGLYCERIDE CONTENT OF SERUM AND ON CONCENTRATION OF TOTAL CHOLESTEROL IN THREE SERUM LIPOPROTEIN CLASSES IN HYPERLIPEMIC SUBJECT L.W.

shown in Figure 4. In the affected siblings such loads of fat resulted in inordinate increases in serum triglyceride concentration, which persisted for two or three days. Smaller, but significant increases occurred in cholesterol and phospholipids (Figure 4). In Subject L.P. even greater changes took place after ingestion of fat in the form of corn oil. Triglyceride concentration rose from 270 to 1,190 mg per 100 ml in 8 hours and to 1,340 mg in 24 hours; after 48 hours it had dropped to 500 mg per 100 ml. These effects resulted from alterations in the amount of very low density lipo-

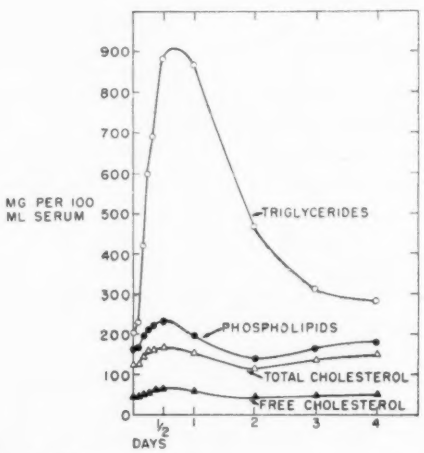

FIG. 4. EFFECT OF A SINGLE MEAL CONTAINING 1.5 G OF CREAM FAT PER KG OF BODY WEIGHT ON SERUM LIPID CONSTITUENTS IN HYPERLIPEMIC SUBJECT L.P. Subject's diet throughout test period contained less than 0.1 g of fat per kg of body weight per day.

proteins; the only other change was a small increase (10 mg per 100 ml or less) in phospholipids of high density lipoproteins.

Effects of administration of heparin

Subject L.P. was given 230 mg of heparin intravenously over a 12 hour period, followed by subcutaneous injections of 200 mg of heparin in aqueous solution (200 mg per ml) every 12 to 24 hours for seven days (Days 50 to 57, Figure 1). His diet during this time contained 1 g of fat per kg. Serum triglyceride concentration decreased slightly after the first three days of the experiment. The decrease appeared to be caused by heparin; it was, however, associated with a slight loss of weight, and no further change occurred in the three days after cessation of heparin during which intake of fat was constant.

Plasma free fatty acid (FFA) concentration

Plasma FFA concentrations were determined in samples taken after the subjects had fasted overnight. The concentrations in sera from the three brothers and the two other hyperlipemic subjects were similar and showed no relationship to serum triglyceride concentration (Table III). Similar determinations were made on two separate occasions on samples taken 10 minutes after intravenous administration of 1 mg of heparin per kg to the hyperlipemic subjects. The increases in plasma FFA concentration were much less in the three brothers than in the other hyperlipemic subjects. In two healthy subjects and the mother of the brothers, increases in FFA concentration after administration of heparin were similar to those of the other hyperlipemic subjects.

Removal of very low density lipoprotein from the circulation

To determine whether the mechanism for removing newly ingested fat from the circulation was impaired in the three brothers, the following experiment was performed. L.P. was given a high fat diet for three days; 2 U of blood was then drawn in acid-citrate-dextrose. The plasma contained 5,200 mg of lipid per 100 ml (81 per cent triglycerides), of which 98 per cent was contained in very low density lipoproteins. Two days before this plasma was obtained from L.P., a 23 year old healthy volunteer and J. P. were started on diets

TABLE III

Effect of administration of heparin on plasma free fatty acid (FFA) concentration

Subject	Heparin	Triglyceride fatty acids	FFA	Increase in FFA after heparin
	mg/kg body wt	*mEq/L*	*mEq/L*	*mEq/L*
Normal				
R.H.	0.2	4.2	0.31	0.50
	1.0	3.7	0.54	0.31
R.G.	1.0	4.3	0.37	0.39
Idiopathic hyperlipemia				
L.W.	1.0	24.7	0.73	0.81
	1.0	37.6	0.55	0.69
F.M.	1.0	91.6	0.75	0.52
	1.0	35.3	0.48	0.49
P. family				
D.P.	0.2	5.0	0.90	0.39
L.P.	1.0	10.8	0.26	0.02
	1.0	9.3	0.29	0.09
J.P.	1.0	13.7	0.31	0.08
	1.0	96.6	0.37	0.18
P.P.	1.0	31.1	1.03	0.17
	1.0	16.0	0.34	0.03

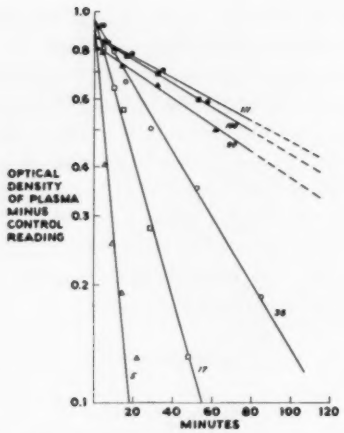

FIG. 5. CHANGES IN OPTICAL DENSITY OF PLASMA FOLLOWING INTRAVENOUS ADMINISTRATION OF LIPEMIC PLASMA FROM L.P. TO HIS HYPERLIPEMIC BROTHER, J.P. (CLOSED SYMBOLS) AND TO A HEALTHY VOLUNTEER (OPEN SYMBOLS). Squares, no pretreatment; triangles, after 0.5 mg of heparin per kg body weight; circles, after 2 mg of protamine sulfate per kg of body weight. Numbers indicate half-times of removal.

containing 0.1 g of fat per kg. On the fourth day of the diet both were given plasma from L.P. (1 ml per kg) intravenously over a 2 minute period; serial blood samples were taken during this time and chilled immediately in ice water. On successive days the procedure was repeated after intravenous administration of 0.5 mg of heparin and 2 mg of protamine sulfate per kg, respectively. Measurements of optical density of the plasma are shown in Figure 5. The turbid lipoproteins were removed rapidly from the circulation of the healthy volunteer; the rate of removal was significantly reduced by administration of protamine. The rate of removal after injection of heparin was much more rapid, although part of the decrease in optical density probably resulted from continuing lipolysis in the drawn blood. In J.P. the rate of lipoprotein removal was much slower and did not appear to be affected by either heparin or protamine. Similar results were obtained in the first transfusion experiment by determination of serum triglyceride concentration in both subjects.[4]

[4] Experiments in dogs have also shown that changes in optical density and triglyceride concentration of plasma after intravenous administration of chylomicrons parallel each other.

Since these measurements are indirect and require subtraction of an appreciable baseline value, the results were considered less precise as an index of the rate of removal of turbid lipoproteins from the blood.

Plasma lipoprotein lipase activity

The preceding studies on the affected brothers suggested either that the lipoprotein lipase liberated by heparin was unable to effect lipolysis of plasma triglycerides or that heparin failed to release the enzyme from body tissues. To determine the cause of the subjects' failure to respond to heparin, the following experiments were carried out.

Substrate studies. While Subject L.P. was receiving the low fat diet, he and a healthy volunteer were given 0.2 mg of heparin per kg intravenously; blood was drawn 10 minutes later. Two aliquots of plasma from each subject were incubated, one with a saline suspension of chylomicrons isolated from pooled serum obtained from the American Red Cross and one with a saline suspension of very low density lipoproteins obtained from L.P. when he was on his usual diet. As shown in Figure 6, both substrates cleared readily

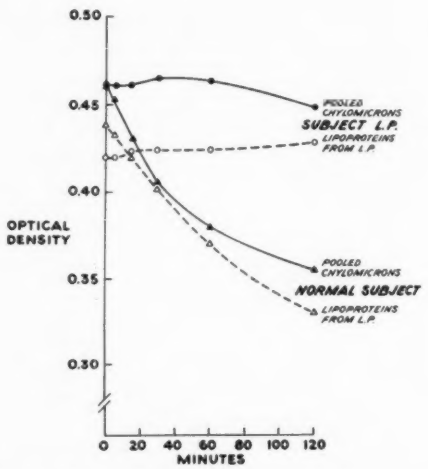

FIG. 6. CHANGES IN OPTICAL DENSITY OF TURBID LIPOPROTEINS DURING INCUBATION WITH POST-HEPARIN PLASMA FROM HYPERLIPEMIC BROTHER L.P. AND A NORMAL SUBJECT. Each incubation tube, containing 0.1 ml plasma and 0.9 ml of a saline suspension of substrate, as noted, was incubated at 37° C; optical density was measured at 500 mμ.

in plasma from the healthy volunteer, whereas neither cleared in plasma from L.P. Similar results were obtained with coconut oil emulsion as substrate on a separate occasion. L.P. was given 1 and the volunteer 0.2 mg of heparin per kg. The standard method of incubation was used. In one hour, 0.50 μmole of glycerol was produced by post-heparin plasma from the healthy volunteer and optical density decreased 0.283 unit. Under the same experimental conditions no glycerol production was detected in post-heparin plasma from L.P. and optical density increased 0.008 unit.

Inhibition studies. To determine whether inhibitory substances in the plasma of the hyperlipemic subjects were preventing optical clearing of triglyceride-rich substrate, the following experiments were performed. Blood samples were drawn before and 10 minutes after administration of 1 mg of heparin per kg to Subjects J.P. and P.P. (lipemics) and of 0.2 mg per kg to D.P. (not affected). Aliquots of both plasma samples were incubated with post-heparin plasma from D.P. as shown in Figure 7. Both pre- and post-heparin plasma from J.P. and P.P. inhibited optical clearing in post-heparin plasma from D.P. The degree of inhibition decreased with time.

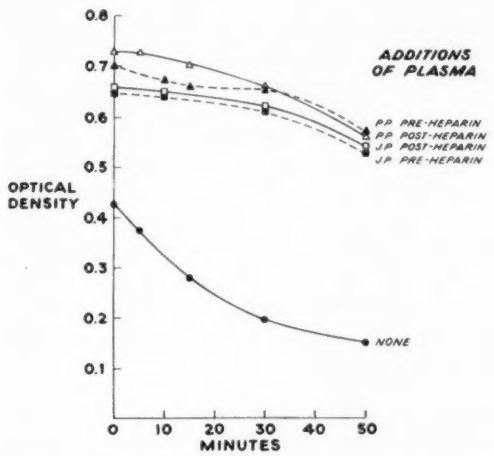

FIG. 7. INHIBITORY EFFECT OF PLASMA FROM HYPERLIPEMIC SUBJECTS ON OPTICAL CLEARING OF COCONUT OIL EMULSION BY POST-HEPARIN PLASMA. Each colorimeter tube contained 0.1 ml of a 0.5 per cent coconut oil emulsion, 0.1 ml of 1 per cent disodium ethylenediamine tetraacetate and 0.25 ml of post-heparin plasma from D.P. (mother); 0.25 ml of pre- or post-heparin plasma from J.P. or P.P. (hyperlipemic sons) was added as indicated, plus saline-phosphate buffer to give a final volume of 1 ml.

A study was then undertaken to determine whether the inhibition resulted from competition between coconut oil and relatively nonturbid, triglyceride-rich lipoproteins in the plasma of the hyperlipemic subjects for the lipoprotein lipase of the post-heparin plasma of D.P. Post-heparin plasma was obtained from hyperlipemic subject L.W. at a time when his plasma (postabsorptive) was only slightly opalescent. A portion of the plasma was centrifuged for 30 minutes at 137,000 × G to remove most of the very low density lipoproteins. Aliquots of the clear infranatant plasma and the uncentrifuged plasma were incubated with coconut oil emulsion. Measurements showed that optical clearing in 60 minutes was 0.215 unit in the tubes containing the infranatant plasma and 0.136 unit in the tubes containing the uncentrifuged plasma; glycerol production was 0.50 and 0.62 μmole, respectively. Thus, lipolysis was not impaired in the presence of very low density lipoproteins as measured by liberation of a product of triglyceride hydrolysis, whereas optical clearing was inhibited considerably. To demonstrate inhibition of optical clearing in post-heparin plasma of a healthy subject by very low density lipoprotein from plasma of a hyperlipemic subject, the following experiment was performed. An aliquot of plasma taken from L.W. before administration of heparin was similarly cleared of very low density lipoproteins. Aliquots of the clear infranatant plasma and uncentrifuged plasma were incubated with plasma obtained from a healthy volunteer 10 minutes after injection of 0.2 mg of heparin per kg. In addition, the post-heparin plasma was incubated alone and with plasma from another healthy subject. As shown in Figure 8, uncentrifuged plasma from L.W. caused the same initial inhibition of optical clearing as that produced by plasma from L.P. and J.P. Clear infranatant plasma from L.W., however, caused only slight initial inhibition of clearing, similar to that produced by plasma from a healthy subject.

Production of free fatty acids in vitro. Since optical clearing proved to be an inadequate measure of lipoprotein lipase activity in hyperlipemic plasma containing turbid lipoproteins, further studies were carried out using production of FFA as a measure of enzyme activity. Pre- and post-

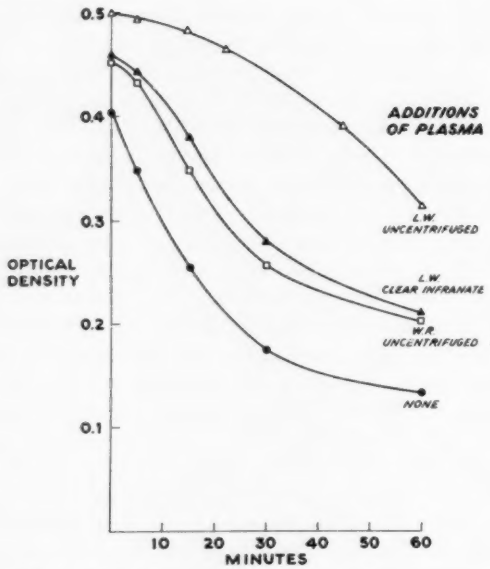

FIG. 8. EFFECT OF ADDITION OF PLASMA FROM HYPERLIPEMIC SUBJECT L.W. AND NORMAL SUBJECT W.R. ON OPTICAL CLEARING OF COCONUT OIL EMULSION BY POST-HEPARIN PLASMA. Experimental conditions were as described for Figure 7.

heparin blood samples were taken from the subjects as described previously. Duplicate 2 ml samples of plasma were mixed with 0.2 ml of 0.5 per cent coconut oil emulsion. Both samples were extracted for FFA analysis, one immediately and the other after incubation at 37° C for 15 minutes. As shown in Table IV, production of free fatty acids was much less in the sera of the three brothers than in the sera of the other hyperlipemic subjects or normal individuals. A study was then carried out to determine the effects on fatty acid production of mixing pre- and post-heparin (0.5 mg per kg) plasma of L.P. and a healthy individual. As shown in Table V, after 15 minutes' incubation, pre-heparin plasma from L.P. caused no inhibition of fatty acid production in post heparin plasma from the healthy individual, although optical clearing was considerably inhibited. Addition of pre-heparin plasma from the healthy individual to post-heparin plasma from L.P. failed to increase the production of fatty acids.

TABLE IV

Production of free fatty acids during incubation of plasma in vitro with coconut oil emulsion

Subject	Increase in free fatty acids	
	Pre-heparin	Post-heparin
	mEq/L	
Normal		
R.H.	−0.03	1.67
R.G.	+0.02	1.81
Idiopathic hyperlipemia		
L.W.	+0.04	2.30
F.M.	−0.04	1.80
P. family		
L.P.	+0.02	0.24
J.P.	+0.06	0.54
P.P.		0.37

TABLE V

Lipoprotein lipase activity of post-heparin plasma from L.P. and a normal subject

Additions	Experiment number			
	I	II	III	IV
			ml	
0.5% Coconut oil emulsion	0.4	0.4	0.4	0.4
Plasma				
Normal pre-heparin	1.0			1.0
Normal post-heparin	1.0	1.0		
L.P. pre-heparin		1.0	1.0	
L.P. post-heparin			1.0	1.0
			mμ	
Decrease in optical density	0.149	0.070	0.003	0.000
			mEq/L	
Increase in free fatty acids	1.68	1.57	0.12	0.11

Lipoprotein lipase activity in other members of P. family. Blood samples were taken from the father, the mother, the daughter and one son (A.P.) and from a healthy volunteer 10 minutes after intravenous injection of 0.2 mg of heparin per kg. In all cases the post-heparin plasma caused rapid optical clearing of coconut oil emulsion and equivalent production of glycerol (about 0.5 μmole per hour).

Fatty acid mobilization

Since mobilization of fat stores during caloric restriction had resulted in no increase in serum lipid concentrations, studies were undertaken to determine whether mobilization of FFA was carried out normally in the hyperlipemic subjects. FFA concentration was no different in L.P., J.P., and P.P. than in normal subjects (Table III). Results of oral glucose tolerance tests were normal in all the hyperlipemic brothers except P.P., who

showed no rise in blood sugar after orally administered glucose and a normal curve in the intravenous test. In all subjects FFA concentration fell normally (11) during the glucose tolerance test.

DISCUSSION

The major defect in lipid metabolism in the three siblings described in this report appears to be a greatly diminished ability to remove newly absorbed fat (chylomicrons) from the circulation. The lipoproteins present in increased concentration in their blood had the physical and chemical characteristics of chylomicrons, and their concentration in plasma varied directly with the fat content of the diet. Furthermore, at a time when fasting serum triglyceride concentrations were only slightly elevated, a fat-rich meal produced an abnormally intense and prolonged lipemia; also, the rate of removal of intravenously administered chylomicron-like lipoproteins from the blood was greatly impaired. It was necessary to lower plasma triglyceride concentrations prior to performing these tests to reduce the size of the "pool" of triglycerides with which the absorbed or injected triglycerides were mixing, since an expanded pool might alter the rate of removal of added triglycerides in spite of a normal or even increased total removal rate.

Almost total elimination of fat from the diet of these subjects failed to reduce the concentration of triglycerides to normal. This finding, however, does not establish the presence of an additional defect in lipid transport, since diets high in carbohydrate and low in fat can cause an increase in triglyceride concentrations in healthy persons (15). The possibility remains, however, that a defect in removal of endogenously-produced triglycerides from the blood is also present. The most likely source of such endogenous triglycerides is the liver, since in hepatectomized dogs the appearance of labeled triglycerides in plasma after parenteral administration of labeled acetate (16) and long chain fatty acids (17) is practically eliminated.

The cause of the reduced concentrations of low density and high density lipoproteins is not entirely clear. Low density lipoproteins contain, in part, the same protein as very low density lipoproteins (18). In the hyperlipemic subjects in this study, as well as in subjects with other hyperlipemic states (19, 20), the concentration of low density and very low density lipoproteins was inversely related. Thus, the low density lipoproteins may serve, at least in part, as "building blocks" for very low density lipoproteins rich in triglycerides or, alternatively, may be liberated by their breakdown. Also, the protein moiety of high density lipoproteins is known to be contained in chylomicrons (18), so that an inverse relationship between the concentrations of chylomicrons and high density lipoproteins might be expected. In this study, however, high density lipoprotein concentration did not increase when the subjects' diets were low in fat. The higher concentration of high density lipoprotein phospholipids when the subjects' intake of fat was normal or high was similar to the increase noted in healthy individuals after ingestion of fat (6), although less marked.

The effects of heparin in the affected siblings differed greatly from its effects in healthy persons and in other subjects with idiopathic hyperlipemia, as noted in this and other studies (21–25). The very low density lipoproteins in their plasma were indistinguishable from normal chylomicrons in susceptibility to lipolysis, as well as in chemical composition. Also, the rate of removal of these lipoproteins from the blood of a healthy subject was similar to that of chylomicrons injected intravenously in dogs (4). In the other hyperlipemic subjects the very low density lipoproteins had a higher proportion of constituents other than triglycerides, and mean particle size presumably was smaller. Such lipoproteins probably would be cleared from the circulation more slowly (26) than those of the affected brothers and might be less susceptible to lipolysis, as in the case described by Carlson and Olhagen (25).

The present studies demonstrated that clearing activity of plasma is an unsatisfactory measure of the lipolytic activity of post-heparin plasma containing appreciable quantities of very low density lipoproteins which are less turbid than the substrate used for the test. This phenomenon was first pointed out by Brown, Boyle and Anfinsen (27). Defective lipemia clearing activity in post-heparin plasma has been noted in a variety of hyperlipemic states; for example: in hyperlipemia induced in rats by administration of alloxan (28);

in rabbits by administration of uranium acetate (29) and cortisone (30) and after excessive bleeding (31); and in man, in the nephrotic state, glycogen storage disease and idiopathic hyperlipemia (32). Klein and Lever, in a series of studies (32–34), found that the inhibitory material was associated with very low density lipoproteins. They also reported that after administration of heparin, serum glycerol levels rose less in hyperlipemic subjects than in normal subjects. Robinson and Harris (31), however, found no inhibition of glycerol production in post-heparin plasma of rabbits after excessive bleeding, and Day and Peters (30) noted that free fatty acid levels were higher in post-heparin plasma from rabbits made hyperlipemic by cortisone than in similar control samples, despite marked inhibition of *in vitro* clearing in the hyperlipemic plasma. Our studies have demonstrated clearly that the inhibition of optical clearing observed in post-heparin plasma of some subjects with idiopathic hyperlipemia results from the presence of relatively large quantities of very low density lipoproteins and is not associated with deficient lipoprotein lipase activity as measured by production of free fatty acids. Since it has been established that lipoprotein lipase forms an enzyme-substrate complex with very low density lipoproteins, it is quite probable that relatively nonturbid very low density lipoproteins in the plasma of hyperlipemic subjects on fat-restricted diets would effectively compete with added substrate for the enzyme. Such lipoproteins are always present in small quantities in blood plasma of healthy humans and in somewhat greater amounts in plasma of patients with manifest coronary heart disease. Similar inhibition of optical clearing without inhibition of fatty acid production has been observed in post-heparin plasma of patients with coronary heart disease, although to a lesser degree than in idiopathic hyperlipemia (35). It appears unwise, therefore, to base estimates of lipoprotein lipase activity of post-heparin plasma on measurements of optical clearing.

In the affected siblings studied here, defective lipolysis in post-heparin plasma, as measured by production of glycerol and free fatty acids *in vitro*, was striking. In addition, these studies showed that this defect did not result from the presence of inhibitors of lipoprotein lipase activity or from lack of a cofactor necessary for lipolysis. We therefore concluded that heparin failed to release lipoprotein lipase from tissue sites and that the enzyme in tissues was probably abnormal or greatly reduced in quantity. When adequate methods for assaying lipoprotein lipase in human tissues, particularly adipose tissue, become available, this problem can be attacked directly.

The present studies cast further light on the normal function of lipoprotein lipase. They strongly support the concept that this enzyme plays an important role in the removal of chylomicron triglycerides from the circulation. They cast considerable doubt on the concept that it promotes hydrolysis of triglycerides in adipose tissue, with formation of FFA for release into the circulation. Instead, our data suggest that another enzyme may carry out this function. This hypothesis is understandable if lipoprotein lipase is assumed to be located at the capillary wall (1, 36) or cell surface and another lipolytic system in the cytoplasm of the adipose tissue cell.

The removal of chylomicrons from the circulation of the liver probably does not involve lipoprotein lipase activity (1). It is likely, therefore, that in the affected siblings the liver is the major site of removal. This could account for fatty infiltration of the liver found in some subjects with idiopathic hyperlipemia. The findings of splenomegaly and fatty infiltration of the bone marrow could be explained by phagocytosis of some of the chylomicrons by reticuloendothelial cells.

In contrast to the defective removal of triglycerides from their blood, the metabolism of free fatty acids in the affected subjects appeared to be normal. In *L.P.*, the lack of increase in serum triglyceride concentration when dietary fat and calories were restricted suggests that fatty acids were being mobilized from adipose tissue as free fatty acids, in accordance with modern concepts of fatty acid transport, rather than as triglycerides.

It is clear that in most hyperlipemic subjects, like the others studied here, release of lipoprotein lipase into the circulation after administration of heparin is not impaired. The nature of the defect in lipid transport in such subjects is not clear, but the finding that the lipolytic activity of their plasma was normal after administration of heparin does not necessarily imply that the enzyme was normally active in body tissues. The present findings, however, strongly suggest the existence of

more than one causative factor for the clinical syndrome of idiopathic hyperlipemia. The affected siblings in this study differed from other patients with idiopathic hyperlipemia not only in their inability to release lipoprotein lipase into the circulation after administration of heparin, but in a number of other ways (see Appendix). Their lipemia could be characterized as a pure "chylomicronemia"; they had pretibial ulcers but no xanthomata; glucose tolerance was normal; up to the present they have shown no evidence of occlusive vascular disease, despite the presence in their plasma of "chylomicron" concentrations in the range of 3,000 to 5,000 mg per 100 ml for as long as 26 years.

SUMMARY

1. Alterations in lipid transport in blood plasma were studied in three siblings with the clinical syndrome of idiopathic hyperlipemia whose plasma contained abnormal concentrations of very low density lipoproteins which had the chemical and physical characteristics of chylomicrons in normal individuals.

2. The concentration of triglycerides in the blood plasma varied directly with the fat content of the diet, and single fat-rich meals given when the subjects' dietary fat intake was low produced marked increases in serum triglyceride concentration which persisted for 48 hours.

3. The blood plasma of these subjects showed little lipoprotein lipase activity *in vitro* after administration of heparin in doses as high as 1 mg per kg of body weight, and intensive administration of heparin to one of the siblings did not lower plasma triglyceride concentration significantly. The deficient enzymatic activity *in vitro* did not result from the presence of inhibitors in the plasma of the affected subjects or from the absence of a plasma cofactor necessary for lipolysis. The triglycerides in the very low density lipoproteins in their plasma were hydrolyzed readily by lipoprotein lipase in post-heparin plasma from healthy individuals.

4. Triglycerides contained in the very low density lipoproteins of the plasma of one of the affected siblings were removed much more rapidly from the circulation of a healthy subject than from that of another sibling. Administration of heparin accelerated and protamine sulfate diminished the rate of removal in the healthy subject but not in the hyperlipemic sibling.

5. Evidence was obtained to suggest that mobilization of fat in the form of free fatty acids from adipose tissue was unimpaired in the affected siblings.

6. In two other subjects with the clinical syndrome of idiopathic hyperlipemia, administration of heparin produced normal levels of lipoprotein lipase activity in their blood plasma. In contrast to the three siblings, the composition of the very low density lipoproteins in the plasma of these subjects differed from that of chylomicrons, xanthomata were present, and, in one, glucose tolerance was impaired.

7. The results of these studies suggest that a genetic deficiency of lipoprotein lipase is responsible for the defective removal of triglycerides from the plasma of the three siblings. They also suggest that more than one defect can result in the syndrome of idiopathic hyperlipemia.

APPENDIX

Clinical data

P. family. This Negro family consists of the parents, 1 daughter and 5 sons; 3 siblings died in infancy. Consanguinity was denied on the basis that the parents were raised in different counties. No other relatives live in the area.

Postabsorptive lipid concentrations (in milligrams per 100 ml) in these subjects at the time of the study (1955) and, when two values are given, in 1959, are as shown in Table VI.

The parents and 3 younger children live on a small farm and raise much of their own food. The animal protein and fat in their diets are obtained largely from chicken and eggs. The father is said to have bronchiectasis. The mother has been obese and hypertensive for many years and had a cerebral vascular accident in 1959. The 3 unaffected children are all well. Data on the hyperlipemic sons are as follows:

J.P. has had pretibial ulcers since early childhood, which are usually more extensive in the summer. He had attacks of upper abdominal pain associated with nausea and vomiting frequently during childhood, but has had none during the past 4 years. At the time of this study, slight lipemia retinalis was present, but no corneal arcus or xanthomata. The skin at the sites of previous ulcerations on both shins was atrophic and depigmented. Peripheral pulses were strong and equal. Results of laboratory tests were as follows: urinalysis, normal; hemoglobin, 17.2 g; sedimentation rate (Wintrobe), 6 mm per hour; prothrombin time, normal. Serum total protein was 6.1 and albumin 3.5 g per 100 ml. Sulfobromophthalein excretion and alkaline phosphatase were

TABLE VI

Postabsorptive lipid concentrations

	Age, time of study	Sickle* trait	Total cholesterol	Free cholesterol	Phospholipids	Triglycerides
	yrs				*mg/100 ml*	
W.P. (father)	59	+	275	70	284	190
			260	69	246	80
D.P. (mother)	48	−	281	72	260	50
			245	71	241	90
F.P. (daughter)	31	+	190	49	245	90
C.P. (son)	23	?	208	54	241	120
J.P. (son)	22	+	384	177	373	3,770
L.P. (son)	17	+	238	116	291	2,980
A.P. (son)	14	−	209	53	232	130
P.P. (son)	7	+	425	208	505	4,800
			335	166	325	3,960

* Demonstrated by paper electrophoresis.

within normal limits. Electrocardiogram and X-ray film of the chest were normal.

L.P. was hospitalized at the age of 8 because of ulcerations over both legs and was found to have milky serum. Despite skin grafting the ulcers have never healed completely. A low fat diet was advised but not followed. At the time of this study the patient was thin but well developed. Ocular examination showed ptosis of the left eye (result of an old injury) and marked lipemia retinalis, but no corneal arcus. Lipemia retinalis was severe when serum total lipid concentrations were above 4 g per 100 ml, slight at 3 to 3.5 g and absent below 3 g. After the lipemia had cleared, optic fundi were found to be normal. Examination of the conjunctival microcirculation by Dr. Robert Akers at a time when serum total lipid concentration was about 5 g per 100 ml showed normal circulation and no tendency toward fragmentation or "sludging" of the cellular elements. There were no xanthomata. The patient had a superficial weeping ulcer, 3×4 cm in diameter, over the right pretibial area and a similar ulcer, 6×10 cm in diameter, over the left. Peripheral pulses were strong and equal. The liver edge was palpable just below the costal margin; the spleen was palpable 2 cm below the left costal margin. Urinalysis was normal, except for urine urobilinogen (2.1 Ehrlich units in 2 hours). The patient had a mild normocytic anemia; packed cell volume was 32 per cent; sedimentation rate (Wintrobe, uncorrected) was 51 mm per hour. Serum total protein was 7.9 and albumin 3.7 g per 100 ml. Sulfobromophthalein excretion and bilirubin were within normal limits. Electrocardiogram and X-ray film of the chest were normal. For a 5 month period during this study the patient adhered to a low fat diet; the pretibial ulcer on the right leg healed completely and the ulcer on the left decreased to about half its former size. Three years after the study, he had an attack of abdominal pain lasting 3 days.

P.P. was found to have milky serum at age 2 when he was examined because of the findings in his two brothers. At the age of 3, he developed ulcers over both shins and knees, which healed subsequently. He has had no unusual illnesses. At the time of this study he was well developed and nourished. He had old, healed scars over the elbows, knees and shins. Ocular examination showed severe lipemia retinalis, but no corneal arcus. Optic fundi were entirely normal after clearing of lipemia. The tip of the spleen was palpable. Peripheral pulses were strong and equal. Laboratory tests gave the following results: urinalysis, normal; packed cell volume, 32 per cent; sedimentation rate (Wintrobe, uncorrected), 25 mm per hour; serum total protein, 7.4 and albumin 3.8 g per 100 ml; alkaline phosphatase and bilirubin, normal. An electrocardiogram and X-ray film of the chest showed no abnormalities. Since the termination of the study the patient has had a number of attacks of mild upper abdominal pain.

Comment. The borderline serum cholesterol concentrations in both parents might be attributed to a defect in lipid metabolism, but serum triglyceride concentrations were normal. For the present, it may be assumed that the hyperlipemic brothers are homozygous for a trait carried in single dose by the parents, although a gene mutation cannot be excluded. We have no explanation for the constant occurrence of pretibial ulcers in the affected brothers, although possibly the combination of sickle cell trait and marked hyperlipemia results in local hypoxia, making the skin of this area liable to necrosis after slight trauma.

Other hyperlipemic subjects

F.M., a 44 year old white man, has had skin xanthomata for about 20 years. For 3 years he has also had repeated episodes of fever lasting several days, for which no cause has been found. With each attack there is rapid and complete clearing of his milky serum. Treatment has consisted of dietary fat restriction and administration of thyroid extract, ethinyl estradiol and heparin. For 6 months prior to study he received no treatment, except for irregular restriction of fat intake. At the time of the study he was well developed and slightly obese. Blood pressure was 160/92 (right arm, sitting position). No xanthelasmata were noted; optic

fundi were normal. The liver was smooth and not tender to palpation; its edge was 5 cm below the right costal margin. The spleen was palpable 2 cm below the left costal margin. The patient had a mild acneform eruption over the upper part of his body and several tuberous xanthomata over the dorsal surfaces of the hands. The remainder of the examination was within normal limits. Urine was normal, except for trace reduction. A hemogram was normal. Sedimentation rate (Wintrobe) was 49 mm per hour. Fasting blood sugar was 187 mg per 100 ml. An electrocardiogram and X-ray films of the chest showed no abnormalities.

L.W., a 35 year old white man, developed eruptive xanthomata over the buttocks and forearms 2 years before this study. Hyperlipemia was discovered at that time. Needle biopsy of the liver showed fatty infiltration but no fibrosis. The xanthomata disappeared when dietary fat was restricted. The patient drank alcoholic beverages heavily for 10 years, but stopped completely 8 years ago. For 7 years he has had epigastric pain after meals, which is relieved by milk or alkali. X-ray films have shown a deformed duodenum. At the time of the study he was a large, muscular, slightly obese man. Ocular examination showed slight arteriovenous compression of the retinal vessels, but no corneal arcus. No xanthomata were present. Peripheral pulses were strong and equal. The remainder of the examination was unremarkable. Results of urinalysis and hemogram were normal. Sedimentation rate (Wintrobe) was 20 mm per hour. Serum total protein, albumin, alkaline phosphatase, prothrombin time, bilirubin and lipase were within normal limits. No abnormalities were shown by an electrocardiogram, X-ray film of the chest, Master's test and oscillometric studies of leg circulation.

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DETERMINATION OF TOTAL BODY FAT BY ABSORPTION OF AN INERT GAS; MEASUREMENTS AND RESULTS IN NORMAL HUMAN SUBJECTS *

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The usefulness of the concept of a "fat-free lean mass" (FFLM)[1] has been widely appreciated (2–6). Such a concept involves a consideration of the mammalian body as a sum of two major components: *a*) the body fat, which varies widely from individual to individual and which may also vary in a given individual from time to time depending upon his state of nutrition; and *b*) the FFLM which is of relatively constant weight in a given healthy individual and of relatively constant composition in the various mammalian species except perhaps for the proportion of bone. Since the concentrations of protein, water and electrolytes in the FFLM vary only slightly, it is the difference in fat content which leads to wide variations in these concentrations in the whole body. This observation was made for individual tissues in 1937 by Hastings and Eichelberger (7).

A knowledge of the relative proportions of these two major components of the body is of obvious biological interest. It has been suggested that the FFLM, rather than body weight or surface area, should be used as a primary reference standard for various purposes (8, 9). Similarly, total body fat would seem to be fundamentally involved in the action and distribution of various substances in the body, such as fat-soluble drugs and anesthetics. Moreover, in recent years it has become increasingly apparent that adipose tissues are metabolically active and knowledge of total body fat could add significance to various studies in fat metabolism.

FFLM contains approximately 72 per cent water (10–12); the variation in this concentration is probably small [Keys and Brozek, in a review article (1), feel the standard deviation is about 3 per cent]. Total body water is subject to direct measurement by the dilution of antipyrine (13), deuterium or tritium. From this measurement and a simple calculation (total body water/0.72), an estimate of the FFLM can be obtained. If this value is subtracted from total body weight, the remainder may be assumed to be body fat.

Total body fat measurements have been attempted by various other methods in living animals (1, 14). In recent years, perhaps that most widely used for physiologic investigation has been densitometry, either alone or (14) combined with body water measurements.

Our fundamental purpose in measuring body fat has been to provide a means of studying the water content of the FFLM under conditions of abnormal hydration or nutrition, and the changes that may occur with age. Under such abnormal conditions, the necessary assumptions underlying calculation of body composition from either densitometry or body water measurements, and probably also of the combined measurement, are open to question. It was essential, therefore, to develop a method that would measure fat more directly and largely independently of the size or condition of the FFLM. In 1935, Shaw, and Behnke and their co-workers (15, 16) attempted such an independent method by washout of dissolved body nitrogen. Since nitrogen is quite lipid-soluble, the volume of nitrogen dissolved in body tissues

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[1] "Lean body mass" has been variously defined to include differing small amounts of "essential" lipids. Since "essential" lipids are at most a small fraction of this mass, confusion might be avoided if the term "fat-free lean mass" is used to describe body weight minus total body fat. Total body fat is defined as the weight of all ether-extractable material in the body. It is not to be confused with "adipose tissue" which, in addition to fat, contains variable small amounts of water and other constituents. This definition of FFLM is considered synonymous with the term "fat-free body" used by Keys and Brozek (1).

is largely a function of the amount of fat present. However, the method is subject to appreciable error and is difficult to execute. Utilizing the same principle, many of the errors and technical difficulties of the nitrogen elimination method might be avoided if a nontoxic, inert foreign gas with a much greater fat/water solubility ratio is employed. The widely used and relatively safe anesthetic gas, cyclopropane, was known to be highly fat-soluble. Measurement of the volume of cyclopropane absorbed from a closed respiratory system proved satisfactory for the determination of total body fat in living rats [± 5 per cent compared with extraction of dried tissues with ether (17)]. Cyclopropane absorption in human subjects, however, presents a difficulty. Equilibration of the gas with body tissues was found to be considerably slower than in rats. Small amounts of gas were still being absorbed after eight hours. It became necessary, therefore, to find some way to circumvent this problem.

In the normal subjects, a mathematical procedure has been utilized to extrapolate observed absorption data to obtain equilibrium absorption values and, from these, reasonably accurate values for total body fat. The present report includes experimental observations on a group of normal subjects. The derived values for fat-free lean mass are sufficiently accurate to lend themselves to use in investigation of changes in this body compartment.

METHODS

Apparatus and experimental procedure

A circular rubber diaphragm (D) (Figure 1) with a central aperture somewhat less than collar size was slipped over the subject's head and snugly fitted to the neck just above the larynx. The head was then introduced into a cylindrical Plexiglas helmet (H), the outer border of the diaphragm being brought over the entire lower lip of the helmet and made airtight with a large rubber band (R). Sufficient glycerol was poured into the helmet through a stopcock (A) to form a coating and seal over the diaphragm. All stopcocks were then turned to provide a completely enclosed gas system involving head and neck of the subject, the helmet, a soda lime container (SL), an airtight pump (P) and a Tissot spirometer (SP) adapted with an additional inlet to enable a continuous circular flow of gases. All seals were coated with a glycerol-starch grease, the pump was lubricated with glycerol, and flexible copper (FCT) or Compar plastic tubing was used throughout because of the solubility and diffusibility of cyclopropane into and through rubber (18), most greases and oils and most commercially available plastics (Lesser and Blumberg, unpublished data).

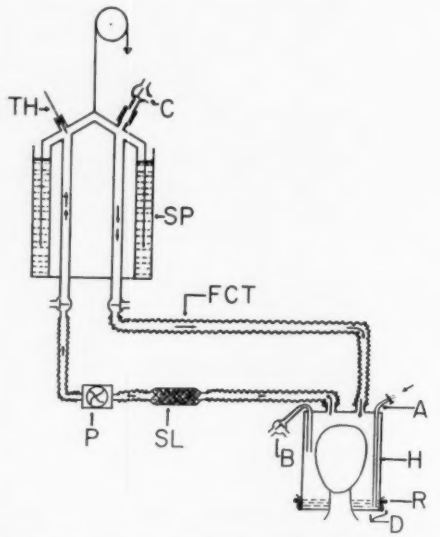

FIG. 1. DIAGRAM OF CLOSED RESPIRATORY CIRCUIT FOR STUDIES OF ABSORPTION OF INERT GAS IN MAN. See text for explanatory details.

The entire system was flushed and filled with pure oxygen and the Tissot bell brought to the desired level. Gas volumes of the total system were from 18.5 to 21.0 L for experiments done at "small respiratory circuit volume" and from 160 to 250 L for "large respiratory circuit volume" experiments. A known small volume of pure cyclopropane was introduced rapidly from a calibrated gas buret through stopcock C and zero time was assumed to be 30 seconds later (small volume) and 90 seconds later (large volume) than the mid-point of the addition. The initial concentration (between 1 and 2 per cent) was well below anesthetic level and no pharmacologic effects were observed. At stated time intervals thereafter samples were taken from stopcock B into gas burets. Total gas volume of the system and of the subject's lungs had been previously measured and was maintained constant throughout (within 0.2 L) by continued addition of oxygen through stopcock C.

All gas samples were analyzed for cyclopropane content by a modification (17) of the method of Robbins (19, 20). With proper care in the hands of a trained technician, consistent recoveries (± 1 per cent) are obtainable.

Description of calculation

The values necessary for the calculation of body fat from the amount of cyclopropane absorbed were determined as follows.

Gas volume of the closed system including the subject's lungs. A method was developed for accurate estimation of this volume and was carried out for each subject prior to the body fat procedure. It involved flushing the apparatus with oxygen, noting the spirometer wheel reading and temperature, sampling the contained gas and then rapidly adding a large known volume of pure nitrogen. After mixing of gases had taken place, temperature and spirometer volume readings were again recorded and samples taken for analysis. "Dead space" volume was then determined in standard fashion:

$$\begin{bmatrix}\text{initial vol.}\\ \text{reading}\\ +\ \text{dead space}\end{bmatrix}\begin{bmatrix}\text{initial}\\ N_2\\ \text{conc.}\end{bmatrix}+\begin{bmatrix}\text{vol. of}\\ N_2\\ \text{added}\end{bmatrix}\begin{bmatrix}\text{conc. of}\\ N_2\\ \text{added}\end{bmatrix}$$

$$=\begin{bmatrix}\text{final vol.}\\ \text{reading}\\ +\ \text{dead space}\end{bmatrix}\begin{bmatrix}\text{final}\\ N_2\\ \text{conc.}\end{bmatrix}.$$

Since the amounts of nitrogen involved were large (about 60 L), the error due to loss or gain of dissolved body N_2 was insignificant. A minor correction for this was made in the calculation. Repeated volume determinations with the same subject in the system agreed within 0.2 L, and agreed closely with the dead space air volume of the apparatus alone, adding the measured functional residual air of the subject and subtracting the volume of the subject's head and neck. To the "dead space" volume determined in this fashion, the volume of gas in the bell of the Tissot spirometer was added, the total being termed the "respiratory circuit volume."

Volume of cyclopropane added to system at beginning of experiment. Standard calibrated gas burets were used throughout. Between 200 and 600 ml of pure cyclopropane was added, depending upon an estimate of the subject's fat content and upon the experimental respiratory circuit volume.

Pressure. The pressures in various portions of the closed system with a subject in position were measured on numerous occasions. Due to the action of the pump, gases contained on the "low pressure" side of the system (soda lime container, helmet, subject's lungs and connecting tubing) remained at 2.5 mm Hg below atmospheric pressure. Gas in the spirometer was always at approximately atmospheric pressure, the average pressure for the entire system therefore being approximately 1.5 mm Hg below atmospheric pressure at all times.

Temperature. Temperature of the gases in the respiratory system was recorded at each sampling time. Temperature of each sample was recorded when reading of buret volume was made.

Solubility coefficient. The solubility coefficients for cyclopropane in fat were determined in a Warburg apparatus and corroborated by a tonometer method and also by extraction of gas from fat samples previously equilibrated with pure cyclopropane. The solubility coefficients (ml gas STP per g fat) for cyclopropane are 10.84 at 37° and 11.81 at 34° C. These studies were done on ether extracts of rat carcasses (21) and have since been repeated on a few samples of human fat, essentially similar values being obtained (Nidus and Lesser, unpublished observations).

Since gas solubility varies with temperature, mean temperature of body fat must be estimated for each subject. This figure is difficult to obtain with great accuracy, as different portions of the body vary to some extent (22–24). In smaller animals rectal temperature is normally 1 to 2° above that of the body as a whole (23); in man, the temperatures of arterial, mixed venous blood and muscle are 0.5 to 2° below that of the rectum (22, 24). For the calculation in these experiments a solubility coefficient of 11.26 ml STP gas per g lipid was used, corresponding to an average temperature for body lipids of approximately 36° C. An error of 1° in this estimate would result in an error of somewhat less than 3 per cent in the total body fat calculation.

Cyclopropane dissolved in nonlipid tissues. A minor correction must be made in the calculations for cyclopropane contained in body fluids and nonlipid tissues. From the body habitus of each subject an estimate was made of the size of the FFLM. The solubility of cyclopropane in nonlipid tissues was studied and has been discussed in some detail in an earlier publication (17). For purposes of gas solubility, the FFLM was viewed as a polyphasic system containing 29 g of protein per 100 ml of iso-osmotic saline. From available data, it has been estimated that at body pH and 36° C such a system would have a solubility coefficient of about 0.33 ml cyclopropane STP per g of FFLM.

Such estimates give merely an approximation of the cyclopropane absorbed by nonlipid tissues. However, due to the high fat/water solubility ratio for cyclopropane under equilibrium conditions, 85 to 95 per cent of cyclopropane in the normal body is contained in the fat. An error as great as 30 per cent in estimating the size and solubility coefficient of nonlipid tissues would therefore result in an error of only 2 to 5 per cent in the value obtained for body fat.

Calculation of total body fat. Observation of the amount of cyclopropane which remains unabsorbed in the system when gaseous equilibrium has taken place between body tissues and the cyclopropane in the system will be discussed in some detail below.

Once these values have been obtained the calculation itself is relatively simple:

$$\begin{aligned}\text{ml cyclopropane at STP absorbed by the subject at equilibrium} &= \text{ml cyclopropane at STP added to the system at zero time}\\ &- \text{ml cyclopropane at STP remaining in the system in free gaseous phase at equilibrium.}\end{aligned}$$

Correcting the total volume of cyclopropane in the animal for that contained in nonlipid tissues:

$$\begin{matrix}\text{ml cyclopropane}\\ \text{at STP absorbed}\\ \text{by fat}\end{matrix} = \begin{matrix}\text{ml cyclopropane}\\ \text{at STP absorbed}\\ \text{by the subject}\end{matrix} - \begin{matrix}\text{solubility coefficient for}\\ \text{nonlipid tissues at body}\\ \text{temperature and partial}\\ \text{pressure cyclopropane under}\\ \text{equilibrium conditions}\end{matrix} \times \begin{matrix}\text{weight of}\\ \text{nonlipid}\\ \text{tissues}\end{matrix}$$

$$\left[\begin{matrix}\text{solubility of cyclo-}\\ \text{propane per g fat}\\ \text{at body temperature}\end{matrix}\right] \times \frac{\text{partial pressure cyclopropane in subject's lungs at equilibrium}}{760} = \begin{matrix}\text{ml cyclopropane STP}\\ \text{per g fat at}\\ \text{equilibrium}\end{matrix}$$

$$\text{Total fat of subject (g)} = \frac{\text{ml cyclopropane STP absorbed by fat}}{\text{ml cyclopropane STP/g fat at equilibrium}}.$$

Corrections to STP are made throughout. Correction is also made for the small differences in cyclopropane concentration between the lungs and the external portion of the respiratory circuit, due to differences in water vapor and nitrogen concentration in these two regions. The assumption in these calculations that the cyclopropane-fat system obeys Henry's law is supported by the results obtained by various methods of solubility estimation (20, 21).

In calculating fat values for the present experiments, the preceding equations and corrections were combined into a single formula, discussed in detail in the Appendix.

OBSERVATIONS

Cyclopropane absorption has been studied in 14 normal subjects by the method described; 26 experiments have been completed. In 11 of these, absorption was observed over approximately an 8 hour period. The absorption data of these prolonged experiments are tabulated (Table I) and discussed below.

The effect of body fat content on the rate and extent of absorption is readily apparent in three subjects of different body weight and habitus, who were studied over a shorter time period.

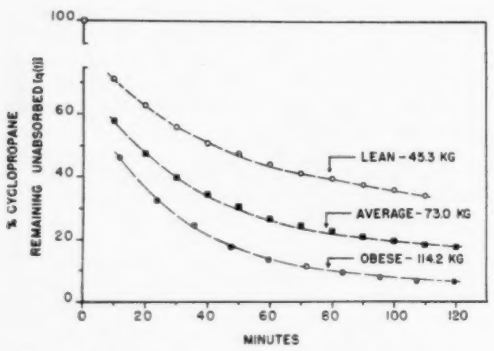

FIG. 2. ABSORPTION OF CYCLOPROPANE BY THREE SUBJECTS OF DIFFERENT BODY WEIGHTS AND HABITUS. Experimental procedure was the same in all instances.

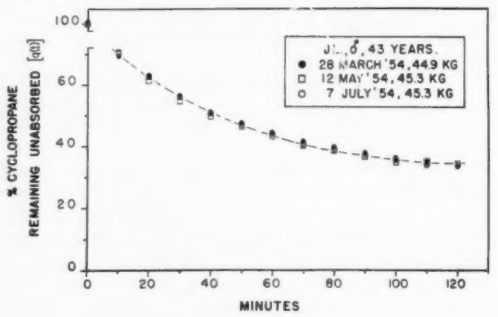

FIG. 3. REPRODUCIBILITY OF CYCLOPROPANE ABSORPTION IN A SUBJECT AT CONSTANT WEIGHTS. Experimental procedure was the same in all instances.

After 110 minutes of absorption from a closed system a small lean man absorbed 65 per cent, a tall average man absorbed 75 per cent, and a small, very obese woman absorbed 95 per cent of the cyclopropane initially added (Figure 2).

The absorption curves in a given subject at con-

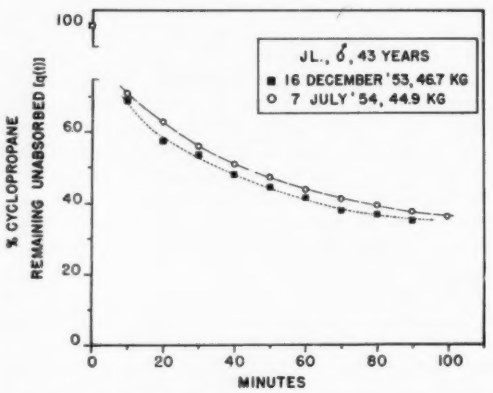

FIG. 4. CHANGE IN CYCLOPROPANE ABSORPTION IN A SUBJECT WITH A SMALL CHANGE IN BODY WEIGHT. Experimental procedure was the same in both instances.

TABLE I

Observed cyclopropane uptake by human subjects; percentage of total cyclopropane in system remaining unabsorbed, q(t), at time t

t	Subjects, age and sex					
	VL, 19, F	GK, 38, F	TJ, 32, M	FK, 29, M	EB, 36, M	PD, 30, M
min						
	A. Normal to moderately obese subjects; small respiratory circuit volume (18.5 to 21.0 L)					
0	100.0	100.0	100.0	100.0	100.0	100.0
12	62.6	61.2	58.0	55.5	58.9	61.5
24	*	52.5	45.1	44.7	43.9	49.9
36	42.9	45.7	37.7	37.4	35.5	40.7
48	38.3	41.1	32.6	31.6	29.6	35.6
60	34.3	37.2	28.8	28.3	24.8	28.2
72	31.1	35.2	26.3	24.9	21.7	27.3
84	28.6	32.5	24.5	22.6	20.5	24.5
96	26.2	30.4	22.6	21.0	17.9	22.1
108	24.2	29.1	21.5	19.2	16.7	20.4
120	22.2	27.9	20.3	17.1	15.7	19.1
132	19.4	26.0	19.5	16.8	14.6	18.4
144	†	21.6	18.8	15.6	14.0	17.4
156	19.3	22.8	17.8	15.6	12.7	16.2
168	18.6	22.3	17.4	13.0	12.6	15.2
180	17.8	21.9	17.3	14.2	12.3	15.2
192	16.8	20.5	16.3	14.2	12.0	14.6
204	16.4	19.9	15.9	13.6	11.6	14.0
216	16.0	18.9	15.7		11.6	13.9
228	†		15.2	13.4	11.4	13.3
240	14.6	18.6	15.0		11.1	
252	14.3		15.4	13.1	11.0	12.5
264	12.5	18.0	14.4		10.7	12.1
276	12.5		14.0	11.8	10.3	12.0
288	12.9	17.1	14.0		10.0	11.5
300	12.6		14.0	11.9	9.85	11.1
312	10.2	15.8	13.7		8.62	10.8
324	12.1		13.5	9.1	9.35	10.4
336	12.3	15.8	13.5		9.58	10.3
348	11.8		13.1	10.7	9.54	10.2
360	12.0	15.2	13.2		9.36	10.1
372	8.62		13.0	10.2	9.49	9.79
384	11.6	14.6	12.9		9.39	9.52
396	10.7		12.6	11.5	8.72	9.24
408	11.0	15.0	12.7			9.43
420	11.1		12.7	10.9	8.79	9.17
432	10.4	14.1	12.2		9.10	9.09
444	10.9		12.1	10.7	8.85	8.92
456	10.9	13.7	12.0		8.65	8.66
468	10.6		11.9	10.7	8.44	8.54
480	10.5	13.8	12.0		8.46	7.85
492			12.0	9.95	8.37	8.49
504		13.4	11.8		8.39	8.36
516				10.1	8.29	8.29
528					8.30	8.07
540				9.23		
564				9.77		
588				9.28		
612				9.33		

t	Subjects, age and sex				
	VL, 20, F	GK, 39, F	TJ, 33, M	RP, 30, M	PD, 31, M
min					
	B. Normal to moderately obese subjects; large respiratory circuit volume (162.9 to 251.7 L)				
0	100.0	100.0	100.0	100.0	100.0
12	88.9	88.5	95.6		92.9
24	84.9	87.0	93.2	90.2	91.4
36	83.5	83.9	93.3	82.5	
48	82.3	81.1	87.1	82.6	87.3
60	78.5	79.9	86.2		83.0
72	78.3	78.8	†	80.1	84.3
84	77.5	76.9	†	81.5	82.7
96	76.9	75.3	83.7	78.9	81.5
108	74.7	73.2	83.8	73.8	79.9
120	72.4	72.9	80.6	77.3	79.8
132	72.0	73.1	81.3	78.3	79.5
144	72.1	71.6	78.8	77.7	78.1
156	70.6	70.1	80.0	69.4	76.3
168	70.0	69.3	79.8	74.6	76.1
180	70.8	68.4	77.4		76.6
192	70.1	68.3	76.8	64.7	74.9
204	70.0	67.3		71.6	73.7
216	68.9	67.1	76.9	73.3	74.3
228	68.1	66.0	74.4	72.1	73.3
240	67.8	65.6	73.9	69.1	72.2
252	66.9	64.2		68.3	71.5
264	67.2		72.4		
276	66.9	62.9	73.3	57.6	72.0
288	65.1	61.5	70.8	63.6	70.7
300	65.2	62.1	71.6	59.9	69.3
312	66.4	61.5	72.3		69.1
324	62.2			59.2	
336	63.8	60.2		67.0	67.7
348	62.1	59.5		65.8	67.5
360	61.5	58.2	67.4	50.1	68.1
372	61.8	58.6	67.7	64.0	67.5
384	61.2		69.1		
396	60.0	58.0	63.4	65.1	65.7
408	59.5	53.9	68.1	63.4	65.2
420	55.9	57.0	61.9	63.4	64.8
432		57.4	67.2	62.3	64.6
444			66.3		
456	60.1	56.7	64.9	64.0	63.4
468	57.1	56.2	64.1	61.7	62.8
480	57.5	55.6		62.3	62.4
492	56.7	55.5		61.5	61.8

* Probable experimental error.
† Known experimental error. Blank spaces denote no sample taken.

stant weight are reproducible within very narrow limits. For example, one subject, when studied on three occasions over several months at weights of 44.9, 45.3 and 45.3 kg, demonstrated variability of cyclopropane uptake of less than 2 per cent (Figure 3). A similar degree of reproducibility was observed in any subject restudied under similar conditions. Subject JL was also studied at a slightly higher weight, presumably reflecting increased body fat content. This change appeared

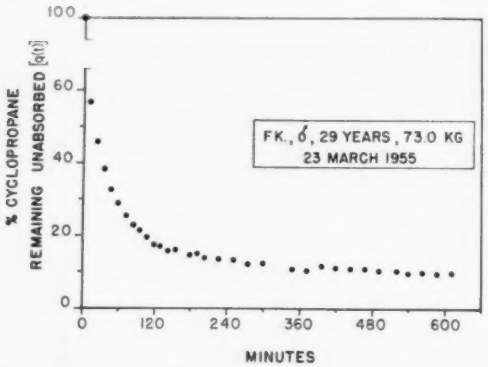

FIG. 5. PROLONGED ABSORPTION OF CYCLOPROPANE BY A NORMAL SUBJECT.

to be sensitively reflected in the rate of cyclopropane absorption (Figure 4).

However, while gaseous equilibrium had essentially taken place between the environment and body tissues in the rat in 90 to 120 minutes (17), all human subjects were still absorbing cyclopropane after an 8 hour period (Table I and Figure 5).

Additional evidence of failure to reach essential equilibrium may be obtained by comparing the values obtained for total body fat by cyclopropane absorption with those for total body fat as calculated indirectly from total body water measurement, F(tbw),[2] in the same subjects. If the observed value at 480 minutes on each absorption curve is assumed to be the "equilibrium" value and is used to calculate total body fat for the subject, F(480), in every instance a smaller estimate of body fat is made by cyclopropane absorption than by F(tbw). In normal to moderately obese subjects, the ratios between the values observed, F(480)/F(tbw), vary from 0.56 to 0.93 (Table II). Similar analysis of these and other "normal" subjects when absorbing gas from a larger respiratory circuit volume showed even greater discrepancy between F(480) and F(tbw) values, indicating that cyclopropane absorption was less complete under these conditions (Table II).

It is therefore evident that an absorption period of as long as 8 hours is insufficient for gaseous equilibrium of cyclopropane to occur in human subjects under these experimental conditions. The discrepancy is systematic and becomes greater with larger volumes of the respiratory circuit. Thus, while ratios of the preceding type may possibly be useful as correction factors to estimate average body fat volumes in large groups of sub-

[2] To avoid repetition of the phrase "total body fat as calculated from total body water" (antipyrine or tritium space), this value will be denoted as F(tbw). It is obtained as $F(tbw) = \text{body weight} - \frac{\text{antipyrine (or tritium) space}}{0.72}$.

TABLE II

Total body fat calculated from "last point" on observed cyclopropane uptake curve compared with total body fat calculated from total body water

	Subject, age, sex	Height	Total body weight	Total body water	Resp. circ. vol.	W_f(tbw)*	W_f(480)†	$\frac{W_f(480)}{W_f(tbw)}$
		cm	*kg*	*L*	*L*	*kg*	*kg*	
Normal fat, small resp. circuit volume	VL, 19 F	161	52.4	28.1	19.6	13.4	12.5	0.93
	GK, 38 F	156	53.3	29.3	21.0	12.6	9.6	0.76
	TJ, 32 M	168	70.1	38.7	19.0	16.3	9.7	0.60
	FK, 29 M	170	73.3	40.4	18.5	17.1	11.5	0.67
	EB, 36 M	183	76.0	39.4	19.7	21.3	15.7	0.74
	PD, 30 M	177	80.3	38.9	18.6	26.3	14.7	0.56
						Mean = 17.83	12.28	0.71
Normal fat, large resp. circuit volume	VL, 20 F	161	51.4	28.1	163.8	12.3	9.0	0.73
	GK, 39 F	156	53.6	29.3	162.9	12.9	9.2	0.71
	TJ, 33 M	168	69.4	38.7	196.5	15.6	6.9	0.44
	RP, 30 M	166	70.5	38.9	213.3	16.5	9.0	0.55
	PD, 31 M	177	80.6	38.9	251.7	26.6	10.5	0.39
						Mean = 16.78	8.92	0.56

* W_f(tbw) = total body fat from antipyrine or tritium space (= total body weight − total body water/0.72).
† W_f(480) = total body fat calculated from cyclopropane absorption, assuming that the 480 minute uptake value is equivalent to the equilibrium absorption (q_∞) value.

jects, their use to estimate accurately the fat content of individuals is not justified.

Since absorption studies cannot feasibly be continued sufficiently long to measure the cyclopropane equilibrium value, q_∞, directly, the problem arose of calculating this value, necessary for the calculation of body fat, from the observed finite portions of the absorption curves actually measured. Such calculation is possible if a physically valid mathematical expression is fitted to the observed absorption curve and then extrapolated for a value of $t = \infty$. A reasonably exact theory of the dynamics of cyclopropane uptake by humans leading to manageable mathematical expressions has so far only been approximated.

Various expressions have been tested. The one utilized to extrapolate the present results conforms with generally held hypotheses for inert gas uptake and transport (25, 26). The quantity of inert gas in the total system (breathing circuit plus subject) is assumed to remain constant throughout. This is supported by evidence that cyclopropane is not significantly metabolized (17) or diffused through the skin (27). The enclosed breathing circuit is assumed to be in contact via the lungs and the arterial blood with the body tissues. The tissues are considered to be grouped into absorption "compartments," each supplied in parallel by the arterial blood (Figure 6). Each compartment is characterized by its capacity for absorbing the particular inert gas ("solubility volume") and by a specific blood flow rate. The compartments, of which the body adipose tissue may be identified as one or more, may not be strictly contiguous anatomical regions, but may be considered as "cyclopropane spaces." The few body tissues which do not conform to such a parallel arrangement probably absorb only small amounts of cyclopropane and should affect the over-all absorption process only slightly.

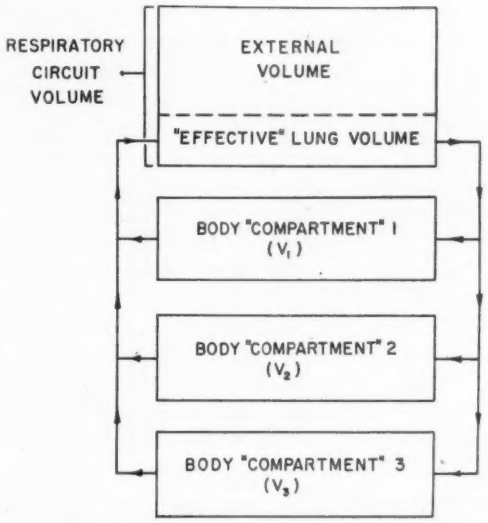

FIG. 6. DIAGRAMMATIC REPRESENTATION OF CYCLOPROPANE UPTAKE BY THE BODY. The system includes a "respiratory circuit volume" (external gas volume of the closed breathing circuit plus an effective lung volume), and some number of "cyclopropane space" body compartments. Each compartment can be defined by a mass, an average solubility coefficient for cyclopropane, and a blood flow.

It is assumed that intratissue diffusion is so rapid in relation to the relatively slower rate of perfusion that intratissue mixing may be considered to be instantaneous, and also that only insignificant diffusion of the inert gas takes place across the surface boundaries of the neighboring tissues. It is further presumed that a steady physiological state exists throughout the course of the experiment. These assumptions permit fitting to the experimental absorption curves an expression of the form:

$$q(t) = q_\infty + a_1e^{-k_1t} + a_2e^{-k_2t} + a_3e^{-k_3t} + \cdots$$

where

$$q(t) = \frac{\text{concentration of cyclopropane in respiratory circuit volume (unabsorbed gas) at time, t}}{\text{concentration of cyclopropane in respiratory circuit volume at t = 0}}$$

and where q_∞ is the limiting value of $q(t)$ as t approaches infinity (see Appendix).

Existing graphical methods of fitting sums of exponential terms to the given curves were found to be inadequate in the present case. Accordingly, a semigraphical method for such fitting was developed and used (28).[3] At each sampling time,

[3] After completion of the present work the attention of the authors was drawn to a paper by Greville (29) in which decay-type data (glucose tolerance curves) were

TABLE III

Total body fat calculated from exponential analysis of observed cyclopropane uptake curve compared with total body fat calculated from total body water

	Subject, age, sex	Resp. circ. vol.	Total body weight	q_{∞}(tbw)*	q_{∞}(exp)†	W_f(tbw)	W_f(exp)	Δ‡
		L	*kg*	%	%	*kg*	*kg*	%
Normal fat, small resp. circuit volume	VL, 19 F	19.6	52.4	10.05	9.75	13.4	13.9	3.7
	GK, 38 F	21.0	53.3	11.1	12.6	12.6	10.7	−15.1
	TJ, 32 M	19.0	70.1	8.05	9.68	16.3	13.0	−20.2
	F.K, 29 M	18.5	73.3	7.46	7.93	17.1	15.9	−7.0
	EB, 36 M	19.7	76.0	6.62	7.30	21.3	18.9	−11.3
	PD, 30 M	18.6	80.3	5.25	6.70	26.3	19.7	−25.1
					Mean =	17.83	15.35	13.57
Normal fat, large resp. circuit volume	VL, 20 F	163.8	51.4	50.3	§	12.3		
	GK, 39 F	162.9	53.6	48.5	§	12.9		
	TJ, 33 M	196.5	69.4	48.2	§	15.6		
	RP, 30 M	213.3	70.5	49.2	§	16.5		
	PD, 31 M	251.7	80.6	42.5	§	26.6		

* Calculated from W_f(tbw), Table II, by Equation [16] in Appendix.
† Extrapolated value by exponential curve-fitting (28).
‡ $\Delta = 100\,[W_f(\text{exp}) - W_f(\text{tbw})]/W_f(\text{tbw})$.
§ Analysis by exponential curve-fitting not possible (see text).

t, the first derivative (i.e., the tangent) of the observed q(t) value may be plotted against the q(t) value itself. This type of plot permits detailed inspection of the experimental data, particularly with reference to the possibility of exponential fitting. If at prolonged time, perhaps 4 to 8 hours of gas absorption, the remaining absorption data fall along a straight line on this plot, it may be assumed that the absorption process can be described as the decay of a single exponential over this time period. The straight line can be extrapolated to the "true" q_{∞} value for such a plot. Deviations from "straightness of fit" indicate either experimental errors or a process inadequately described by the decay of a single exponential term.

A group of six average to moderately obese subjects was studied under low respiratory circuit volume conditions (18.5 to 21.0 L, Table I, A) and the cyclopropane absorption curves were subjected to this semigraphical exponential analysis. After approximately 3 to 4 hours of absorption, the data in each instance seemed to follow a straight-line fit of a single remaining exponential term. In four of the six subjects the fit was very good (example, Figure 7, A), while the other two subjects demonstrated slight "kinks" or "oscillations" of the data about a presumed average straight line (example, Figure 7, B). The lines were extrapolated to obtain q_{∞} absorption values, from which total body fat was calculated.

These straight lines yield not only q_{∞}, but also a (long) "first" exponential, $a_1e^{-k_1t}$. This "peeling off" process was continued, and yielded two further exponential terms within the accuracy of the data.[4] Three exponential terms were therefore adequate to represent the observed absorption curves in each instance. Since the values derived from these terms were of the same order of magnitude in all subjects, it is felt that the "cyclopropane spaces" and exchange rates implied by these exponentials may have physiological significance.

Total body fat derived from the extrapolated values of q_{∞} ranged from 10.7 to 19.7 kg with a mean of 15.4 kg (Table III). These values for

fitted by a constant plus one exponential by the same method used here. Of several curve-fitting methods tried, Greville concluded that for his type of data, which were similar to those of the present paper, the present method was the most satisfactory one.

[4] The "third" exponential term with a half-life of only a few minutes is probably a composite of many very short exponentials, probably including lung and highly perfused organ filling rates. The experimental design permitted only scanty observations over the period required for definition of this exponential, and its values are, at best, a gross average approximation of many rates. In contrast, the much longer "first" exponential term is observed very carefully over many hours, enabling results to be interpreted with greater confidence.

body fat are in the expected range for the size and habitus of the individual subjects. They may be compared with the respective body fat values calculated indirectly from total body water [F(tbw), Table III]. In all but one subject, total body fat from cyclopropane absorption F(exp) was somewhat lower than the F(tbw) value, the mean difference being 2.5 kg of body fat.

The calculation F(tbw) is an indirect one, involving several numerical assumptions and subtraction of two large numbers to yield a small difference. The error of measurement of total body water in these subjects by dilution of antipyrine or tritium is considered to be ± 5 per cent. In addition, there is some variation about a 0.72 value for water content of the FFLM. [Keys and Brozek (1) estimate a standard deviation of 0.02 to 0.03]. However, there is fairly good evidence that in most mammalian species the *mean* FFLM hydration value is close to 0.72. The estimate of total body fat from the calculation: body weight − (body water/0.72) is therefore subject to two variables, both *randomly* distributed. Error analysis of this situation in an individual containing 25 per cent body fat indicates a potential error of about ± 20 per cent in the estimation of fat mass from body water measurement. This error is, however, of a random nature, rather than the directional one observed in the present comparison (27). It is likely, therefore, that total body fat is being somewhat underestimated by cyclopropane absorption under these conditions.

The inability to apply a single exponential fit closely to the data of *large* respiratory circuit volume experiments is understandable on theoretical grounds. Considerations based on a theoretical model of gas uptake by the body (Figure 6) and analysis of the present experimental observations indicate that the rate at which the body absorbs cyclopropane from the environment increases as the volume of the closed respiratory circuit becomes smaller. Therefore, the same subject, under identical experimental conditions except for the respiratory circuit volume, will approach gaseous equilibrium more slowly at larger volumes. A change from the "low volume" conditions employed in the present experiments (18.5 to 21.0 L respiratory circuit volume) to "high volume" conditions (160 to 250 L volume) should increase the half-life of cyclopropane absorption in body fat about threefold. The addition of fat to the body will also increase the time necessary for approach to equilibrium unless the local supply of blood to body lipids increases approximately proportionately to the increase in lipids. If gas absorption is assumed to follow an exponential rate, these considerations and the experimental observations would indicate the *slowest* half-life (essentially describing absorption into the lipids) to be roughly 90 to 180 minutes for average subjects under low respiratory circuit volume conditions and about 360 minutes for average subjects under large respiratory circuit volume conditions. Therefore, the normal or slightly obese person studied over an 8 hour period under low volume conditions, has completed about 3 or 4 half-lives of absorption into his "slowest" compartment, minimizing the effect of any minor deviations from an ideal exponential description and enabling relatively accurate extrapolation to a q_∞ value.

DISCUSSION

The preceding analysis of the observed experimental data is based upon an idealized compartment model of inert gas uptake by the body. The exponential analysis follows, with minor modification, the generally accepted theories of such absorption processes (26, 30). Application of this analysis to the present experimental observations has led to values for total body fat which are reasonable, but systematically somewhat lower than expected. In addition, the observed deviations from ideal exponential rates are often of greater magnitude than experimental errors, suggesting that one or more of the assumptions involved in setting up the model, or in curve-fitting the data, may not be completely valid. Several of these assumptions may be reviewed.

The steady state. In order to successfully extrapolate a situation such as the present one, the processes involved must go on in more or less constant fashion over the period of observation. In the present instance, we would have to assume constancy of transmision of cyclopropane from the external circuit to the alveolar volume and thence across the pulmonary absorbing surface, constancy of cardiac output and, most particularly, constancy of perfusion of adipose tissue. We have been unable to find published information describ-

ing any shunting of blood or variation of blood supply to adipose tissues. Gersh and Still (31) noted only 25 to 50 per cent of local fat capillaries of rats to be "open" under the conditions of their study, but whether the "reserve" capillaries are readily available and whether perfusion may vary under the conditions prevalent in our experiments remains speculative. Random changes in blood supply to one or more large depots of fat during an observed absorption experiment would lead to changes in uptake rates of cyclopropane by the tissues involved, and would complicate the mathematical analytical treatment. The "kinks" and

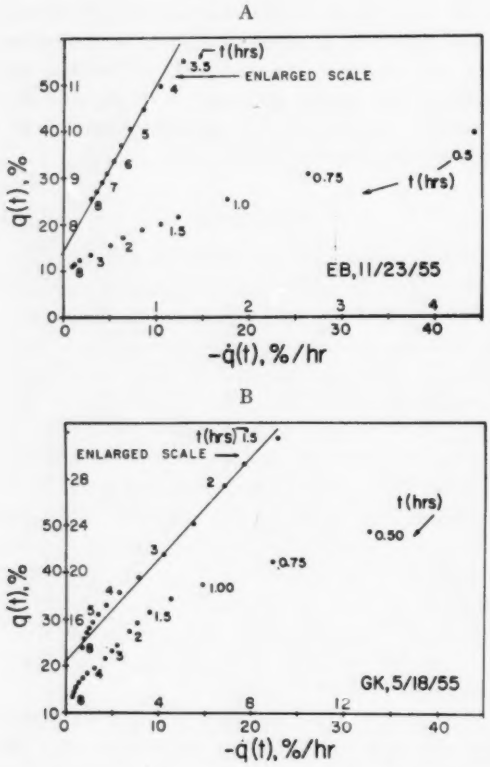

FIG. 7. EXAMPLES OF EXPONENTIAL CURVE-FITTING OF CYCLOPROPANE ABSORPTION DATA ON NORMAL SUBJECTS (28). At each sampling time, the first derivative (tangent) or $-\dot{q}(t)$ is plotted on the abscissa against the observed $q(t)$ value itself (ordinate). A final single exponential value, if present, can be plotted on a straight line to an equilibrium absorption value (q_∞). In each figure the upper points are an enlarged scale of the lower points, and conform to the inner scale on the ordinate.

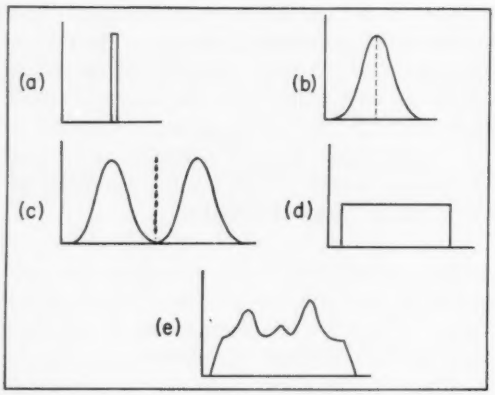

FIG. 8. VARIOUS POSSIBLE SPECTRA OF EQUAL-SIZED SMALL SEGMENTS OF ADIPOSE TISSUE WITH REFERENCE TO THEIR BLOOD SUPPLY. In each diagram the abscissa depicts F_1/V_1, the blood perfusion rate for unit volume of tissue. The ordinate depicts N_1, the number of segments so perfused. See text for discussion.

"oscillations" observed in several of the present exponential analyses could be explained by changes in rate of perfusion of fatty tissues and would be difficult to interpret. To our knowledge this problem has not been seriously considered in the past. The possibility that the present simplified mathematical analysis contains a systematic error due to steady state changes cannot be ignored.

Relationship of exponential analysis to perfusion of adipose tissue. The present extrapolation leading to q_∞ was based upon approximating the observed absorption data by a single exponential function at long time (about 4 to 8 hours). The validity of this approximation must be considered. The total fatty tissue in the body may be viewed as a sum of cubes of, for example, 1 cm^3 volume. This size cube is selected, since a rough analysis suggests that the internal distances are small enough that diffusion may be considered to be rapid *within* the cube, while diffusion of cyclopropane from surrounding cubes should be small in comparison with the cyclopropane taken up from the cube's intrinsic blood supply. Thus, the 1 cm^3 piece of fatty tissue might be considered as a fundamental perfusion unit, in parallel with many similar units. The relative rate at which the cube fills with an inert gas from an infinite external volume may be expressed as:

$$\frac{\text{volume of blood flow per minute through the 1 cm}^3\text{ cube } (F_i)}{\text{total cyclopropane necessary to "fill" the cube at infinite time under conditions of the experiment (volume} \times \text{average solubility coefficient for cyclopropane} = V_i\lambda_i)}.$$

As the blood flow increases or the tissue solubility decreases, the "filling up" time becomes more rapid. Since solubility can be assumed to be roughly similar in all cubes, the λ_i terms are essentially constant and each small segment of fatty tissue can be compared with other such segments solely in terms of relative blood flow rates. In order for all the adipose tissue to absorb cyclopropane at the same rate, each unit would have to be similarly supplied with blood. That this is probably not the case can be inferred from histological examination of subcutaneous, omental, perirenal and other fat depots.

There have been few reports on the blood supply to fatty tissue and even less information is available on relative differences in perfusion rates. If the blood flow to all segments of adipose tissue throughout the body is the same or varies only within a narrow range, the uptake of an inert gas by the total body fat may be described by a single exponential term (Figure 8, a, b). Another possible set of circumstances would be the existence of two or three large groupings of fat units, each group identified by a certain blood flow rate. The single long exponential term derived from the absorption curves of the present experiments would then represent an average of two, or perhaps several, exponential terms which are reasonably closely spaced (Figure 8, c). If the exponential rates are not too far from one another (i.e., the values for blood perfusion are not too dissimilar for the various groups of units) it would be very difficult to separate the exponential terms and they may appear as a single term (32). If, in fact, multiple exponential rates exist for filling of body fat with cyclopropane, the mistaken single exponential analysis would alter the value for q_∞ in the direction necessary to account for the observed systematic discrepancy in body fat values.

Blood perfusion rates of the various segments of fat throughout the body may not lend themselves to grouping about a few values but may instead vary over a wide range (Figure 8, d). Such a situation could not adequately be described by one, or even a few, exponential terms, but rather by a sum of many, or integral of, exponentials with closely spaced half-lives. Such an integral can lead to expressions of, for example, the form $1/t^p$.

Diffusion. The assumption has been made that diffusion of gas from capillary blood through each tissue is so rapid that it causes negligible limitation of over-all uptake and also that insignificant diffusion takes place between neighboring tissues. In contrast with studies on gases with less fat solubility (26, 30), the conditions present when a very highly lipid-soluble gas (such as cyclopropane) is being absorbed may alter the relative importance of perfusion and diffusion in delivery of gas to body lipids. While diffusion of cyclopropane in aqueous solutions is apparently rapid ($D \geqq 10^{-4}$ to 10^{-5} cm^2 per second), it may be slower in living tissues (26). Preliminary studies in this laboratory indicated diffusion constants for cyclopropane in fatty tissues to be 10^{-5} to 10^{-7} cm^2 per second. Diffusion constants of this magnitude are probably not slow enough to cause significant lack of uniformity within an individual depot of fat. However, consideration must also be made of possible diffusion of cyclopropane into fatty tissues from adjacent nonlipid tissues. Without accurate values for diffusion constants of the gas, or for blood perfusion rates, the relative importance of such diffusion cannot be predicted. The analysis of gas diffusion in solving the absorption curves and in extrapolating to q_∞ cannot be carried out by a finite sum of exponential functions. The specific expression to be used would depend upon the diffusion constants and upon the geometric relationships of fat and nonfat tissues, which occur in various shapes and sizes.

Thus, at the present state of knowledge, there are several possible methods of mathematical analysis to describe the uptake of a highly fat-soluble inert gas by body lipids. Each method is based upon one or more simplifying hypotheses as to the manner of transport of the gas to the lipids. More exact analysis must await more

complete knowledge of the geometric arrangement of the various depots of fat tissue, of accurate diffusion constants of the gas through fat tissue and of absolute and relative blood flow rates through various depots of fat. In addition, the existence of a "steady state" of blood flow to body fat must be confirmed, or changes in blood flow measured before more precise analysis is possible.

Despite these gaps in our knowledge, much information can be obtained by the techniques employed. The use of a relatively small respiratory circuit volume (18.5 to 21.0 L) appreciably speeds the rate of absorption of inert gases so that over the 8 hour experimental absorption period a normal or moderately obese human subject has completed approximately three to four half-lives of gas absorption into his "slowest" body compartment. Body fat has therefore taken up 80 to 95 per cent of its potential cyclopropane. It is likely that modifications of the present technique can further accelerate gas uptake, leading to even closer approach to equilibrium gas absorption. The extent of absorption provides a favorable analytic situation for extrapolation to the equilibrium q_∞ value necessary for calculation of total body fat. This is confirmed by the good results obtained in the present experiments carried out under conditions most favorable for rapid uptake of the inert gas.

The initial interest in developing a direct method for estimating total body fat was to obtain independent values for, and to observe changes in, the fat-free lean mass. Since this mass represents most of the body weight, even moderate error in direct estimation of total body fat will lead to only small error in calculation of the FFLM (FFLM = total body weight − total body fat). The FFLM values calculated from F(cyclo) agreed closely with FFLM values obtained by calculation from total body water (Table IV), the average deviation by the two methods being 2.5 kg, or ± 5 per cent.

It would appear, therefore, that a method is available for the direct, independent *in vivo* measurement of total body fat in normal to moderately obese human subjects with reasonable accuracy. From this measurement, accurate values can be derived for FFLM under varying conditions. The method provides a tool which, in conjunction with measurements of total body water, should permit study of the degree of hydration of the FFLM under normal and certain abnormal conditions and throughout the aging process.

TABLE IV

Fat-free lean mass calculated from total body fat by exponential curve-fitting analysis compared with fat-free lean mass calculated from total body water

	Subject, age, sex	W_l(tbw)*	W_l(exp)†	Δ‡
		kg	*kg*	*%*
Normal fat, small resp. circuit volume	VL, 19 F	39.0	38.5	−1.28
	GK, 38 F	40.7	42.6	4.67
	TJ, 32 M	53.8	57.1	6.13
	FK, 29 M	56.1	57.3	2.14
	EB, 36 M	54.7	57.1	4.39
	PD, 30 M	54.0	60.6	12.20
	Mean =	49.72	52.20	5.13
Normal fat, large resp. circuit volume	VL, 20 F	39.0	§	
	GK, 39 F	40.7	§	
	TJ, 33 M	53.8	§	
	RP, 30 M	54.0	§	
	PD, 31 M	54.0	§	

* W_l(tbw) = fat-free lean mass = total body water/0.72.

† W_l(exp) = fat-free lean mass = total body weight − total body fat calculated from exponential analysis of cyclopropane uptake (see Table III).

‡ $\Delta = 100\ [W_l(\text{exp}) - W_l(\text{tbw})]/W_l(\text{tbw})$.

§ Analysis by exponential curve-fitting not possible (see text).

SUMMARY

A method has been presented for measurement of total body fat in human subjects by absorption of an inert, highly fat-soluble gas from a closed respiratory system. Results are highly reproducible and reflect small changes in fat content. In a group of normal to moderately obese subjects, body fat averaged 15.4 kg, about 12 per cent lower than the fat values for the same subjects calculated indirectly from total body water. The present gas absorption procedure and calculation possibly lead to a systematic underestimation of total body fat.

Since equilibrium of body tissues with cyclopropane in the respiratory system is prolonged in human subjects (longer than 8 hours), observed absorption data were extrapolated to obtain equilibrium values necessary for the calculation of total body fat. Accurate extrapolation requires a mathematical formulation which adequately describes the physiological processes involved. The formulation used here involves a sum of exponential

terms and coincides with generally accepted theories of inert gas absorption and transport. Data obtained from the present experiments suggest, however, that various aspects of these theories remain open to question. Further knowledge is required of the distribution and nature of blood supply to adipose tissues, of accurate diffusion constants of gases in living tissues and of possibly changing conditions within a given subject which affect the "steady state" before more exact mathematical description (and extrapolation) becomes possible.

By subtraction of measured total body fat from body weight, values may be derived for fat-free lean mass (FFLM). Except for highly obese subjects, total fat is small in relation to body weight and FFLM, and the relative error in the latter calculated value is therefore smaller. Mean FFLM from body fat was 52.2 kg, FFLM calculated from total body water, 49.7 kg.

By the gas absorption technique, fat is being measured directly and essentially independently of the size or condition of the FFLM. It may be utilized, therefore, in conjunction with measurement of total body water, to study changes in hydration of the FFLM that may occur in pathological states and with age.

APPENDIX

Calculation of total body fat. The experimental data are expressed in terms of the quantity

$$q(t) = \frac{V_N C_e(t)}{Q(t)} \qquad [1]$$

in which $C_e(t)$ is the measured concentration of cyclopropane in the sample drawn at time t from a region of the closed respiratory circuit *external* to the subject (not including the lung volume). This volume will be termed the "external volume". $Q(t)$ is the measured initial amount of cyclopropane introduced into the external volume, minus the measured amount contained in all the samples drawn up to the time t, and V_N is the external volume plus an effective lung volume (defined below). If the experiment were continued for a length of time sufficient for the body tissues to equilibrate with the cyclopropane concentration in the external volume, $q(t)$ would approach an equilibrium value q_∞ given by

$$q_\infty = \frac{V_N C_e(\infty)}{Q(\infty)} \qquad [2]$$

in which $C_e(\infty)$ is defined as the constant concentration of cyclopropane that would exist at sufficiently long time in the external volume and $Q(\infty)$ as the amount of cyclopropane that would exist in the entire system of closed respiratory circuit and subject at sufficiently long time. In practice a value for q_∞ is deduced by extrapolation of the $q(t)$ values measured over a period of approximately 8 hours. The extrapolation is carried out by curve-fitting exponential functions to the data (28). Exponential functions are chosen because they are the mathematical expression of the general compartment model (32, 33) which is assumed applicable to the present experiments.

The constant V_N is a measure of the external volume plus an effective lung volume for containing inert gas. It is determined by nitrogen dilution (see text) and termed the "respiratory circuit volume." The assumption is made that if this determination were carried out with cyclopropane, the resulting respiratory circuit volume would be the same as for nitrogen. This assumption, which is plausible because of the inert nature and comparable molecular weight of both gases, would be expected, even if not valid, to result in only a small error in the effective lung volume. Such an error is well within the over-all error of measurement of the cyclopropane uptake.

In the cyclopropane uptake experiment a conservation principle using the quantities of Equation [2] can now be applied. The principle states that the amount of cyclopropane initially introduced into the external volume equals the amount leaving the respiratory circuit volume, ΔQ_{leav}, plus the amount finally remaining in the respiratory circuit volume, or

$$Q(0) = \Delta Q_{leav} + V_N C_e(\infty). \qquad [3]$$

Now ΔQ_{leav} is the amount which goes into the body, ΔQ_{body} (the body does not include the effective lung volume), plus the total amount withdrawn in the samples during the experiment, ΔQ_{samp}. On the other hand, $Q(0) - \Delta Q_{samp}$ is the amount remaining in the entire system at long time, $Q(\infty)$. Hence, Equation [3] can be written

$$Q(\infty) = \Delta Q_{body} + V_N C_e(\infty). \qquad [4]$$

Substituting Equation [4] into Equation [2] and rearranging terms gives

$$\frac{\Delta Q_{body}}{V_N C_e(\infty)} = \frac{1 - q_\infty}{q_\infty}. \qquad [5]$$

A connection is next sought between the amount of cyclopropane absorbed by the body, ΔQ_{body}, and the amount of fat contained by the body. To this end, it is assumed that the body is composed additively of two components, *a*) total body fat and *b*) everything else, termed fat-free lean mass. By this statement is meant not only that the total body weight W may be written as the sum of a weight of total body fat W_f plus a weight of fat-free lean mass W_l or

$$W_f + W_l = W \qquad [6]$$

but also that the amount of cyclopropane absorbed by the body at long time can be written as the sum of two terms

$$\Delta Q_{body} = W_f C_f'(\infty) + W_l C_l'(\infty) \qquad [7]$$

in which $C_f'(\infty)$, $C_l'(\infty)$ are appropriate average concentrations of cyclopropane per unit weight of fat and fat-free lean mass, respectively. (The prime notation denotes per

unit weight.) Equation [6] can be substantiated, for example, by identifying W_f with the weight of ether-extractable material from a body and W_l with what is left. Equation [7] might be substantiated directly by determining $C_f'(\infty)$, $C_l'(\infty)$ by biopsy measurements of various body tissues and using the values W_f, W_l obtained independently by ether extraction. One could also use the more indirect consequences of Equation [7] as determined below to compare with ether-extracted values. Such comparison has been made for the rat (17), and indicates the validity of Equation [7].

The concentrations $C_f'(\infty)$, $C_l'(\infty)$ of cyclopropane in the body at sufficiently long time, that is, at equilibrium, are next related to the equilibrium concentration $C_e(\infty)$ in the external volume. For brevity the notation (∞) is dropped. All concentrations are assumed to be equilibrium values. Consider first C_f'. Write

$$\frac{C_f'}{C_e} = \frac{C_f'}{C_A}\frac{C_A}{C_e} \qquad [8]$$

where C_A is the equilibrium concentration (per unit volume) of cyclopropane in the alveolar spaces of the lung. The body fat is assumed to be in thermodynamic equilibrium with the gases in the alveolar spaces via the mediation of the circulating blood. Hence, the ratio C_f'/C_A can be expressed in terms of a solubility coefficient α_f' of cyclopropane in fat as

$$\frac{C_f'}{C_A} = \alpha_f'\frac{T_A}{273} \qquad [9]$$

where α_f' is in milliliters STP of cyclopropane per *gram* of fat, and T_A is the absolute temperature in the alveolar volume. The concentration ratio C_l'/C_A can be similarly expressed as

$$\frac{C_l'}{C_A} = \alpha_l'\frac{T_A}{273}. \qquad [10]$$

The solubility coefficients α_f', α_l' per gram of tissue are used here instead of the more conventional Bunsen solubility coefficients α_f, α_l per milliliter of tissue because the former were measured (17, 21), making knowledge of the density of the body fat unnecessary.

The concentration of cyclopropane, C_A, in the alveolar volume, V_A must next be related to the concentration C_e in the external volume.[5] In the alveolar volume V_A, the temperature T_A and water vapor pressure p_{vA} differ slightly from the corresponding quantities T_e, p_{ve} in the external volume V_e. Also, the concentration of nitrogen is somewhat greater in the alveolar volume than in the external volume because the rate of expiration of carbon dioxide is less than the rate of inspiration of oxygen (the respiratory quotient effect) (34). It can be shown, using the perfect gas law, the condition of equal total pressure in external and alveolar volume, and the approximate value for concentration ratio of nitrogen between alveolar volume and external volume $[N_2]_A/[N_2]_e = 80/79$ that

$$\frac{C_A}{C_e} = \frac{80}{79}\frac{T_e}{T_A}\left(\frac{p_e - p_{vA}}{p_e - p_{ve}}\right). \qquad [11]$$

Equations [7] to [11] can now be substituted into Equation [5] to yield

$$\alpha_f'W_f + \alpha_l'W_l = \alpha'W \qquad [12]$$

where

$$\alpha'W = \left(\frac{79}{80}\right)\left(\frac{273}{T_e}\right)\left(\frac{p_e - p_{ve}}{p_e - p_{vA}}\right)\left(\frac{1 - q_\infty}{q_\infty}\right)V_N. \qquad [13]$$

The quantity $\alpha'W$ defined by Equation [13] is expressed in terms of quantities determined by the experiment. The physical interpretation of this quantity is given by the left hand side of Equation [12] as the number of milliliters STP of cyclopropane absorbed by the body if the body is held in solubility contact with a reservoir at body temperature containing cyclopropane at one atmosphere pressure (if W_f, W_l are expressed in grams and α_f', α_l' in milliliters STP cyclo per gram of tissue). Hence, the quantity α' is an average solubility coefficient for the body as a whole.

The solubility equation [12] can now be solved simultaneously with the body weight equation [6] to yield W_f and W_l. Thus,

$$\frac{W_f}{W} = \frac{\alpha' - \alpha_l'}{\alpha_f' - \alpha_l'} \qquad [14]$$

$$\frac{W_l}{W} = \frac{\alpha_f' - \alpha'}{\alpha_f' - \alpha_l'} \qquad [15]$$

The form of Equations [14] and [15] exhibits the analogy between the solubility method and the densitometric method (1) of determining total body fat. It is useful, also, to solve Equations [12] and [13] for q_∞. The result is

$$q_\infty = \frac{V_0'}{V_0' + \alpha_f'W_f + \alpha_l'W_l} \qquad [16]$$

where V_0' is defined as

$$V_0' = \left(\frac{79}{80}\right)\left(\frac{273}{T_e}\right)\left(\frac{p_e - p_{ve}}{p_e - p_{vA}}\right)V_N. \qquad [17]$$

The following solubility coefficients for cyclopropane in total body fat and in fat-free lean mass, at a temperature of $36 \pm 1°$ C, were used:

$$\begin{aligned}\alpha_f' &= 11.26 \pm 0.40 \text{ L cyclo STP per kg fat}^6\\ \alpha_l' &= \left(0.46 \pm 0.10\,\frac{\text{L cyclo STP}}{\text{L body water}}\right)^7\\ &\quad\times\left(0.72 \pm 0.03\,\frac{\text{L body water}}{\text{kg fat-free lean mass}}\right)^8\end{aligned} \qquad [18]$$

[5] From the standpoint of concentration, the respiratory dead space volume is a transitional region between the external volume and the alveolar volume. This dead space volume may be considered, for the present purposes, to be suitably divided between the external and alveolar volumes. It is included in the measurement of respiratory circuit volume.

[6] This value is an average of measurements made on pooled rat fat (21). Later results on fat samples from humans varied only slightly from those on pooled rat fat (35).

[7] Estimated from measurements on various solutions of water, protein and salts (17).

[8] Summary of many experiments critically reviewed by Keys and Brozek (1).

or,

$$\alpha_l' = 0.33 \pm 0.073 \text{ L cyclo STP per kg fat-free lean mass.} \quad [19]$$

Total body fat W_f and fat-free lean mass W_l were calculated by Equations [14] and [15] for those experiments in which q_∞ could be derived. The basic data used in the calculations, aside from the solubility coefficients, Equations [18] and [19], are given in Tables I, II and III. In determining the values of α' by Equation [13] the following temperature and pressure values were used: T_e, p_e average temperature and pressure, respectively, in the last 2 hours of each experiment (maximal deviation from these values less than 1 per cent), $p_{vA} = 47$ mm Hg, $p_{ve} = 0.80 \times$ water vapor pressure at temperature T_e, ($p_{ve} \approx 23$ mm Hg). The results are given in Tables III and IV. The values of q_∞(tbw) in Table III were calculated by Equation [16] using W_l = (total body water)/0.72 and $W_f = W - W_l$.

Accuracy of cyclopropane uptake method. The accuracy of total body fat and fat-free lean mass determination by the cyclopropane uptake method can be estimated by Equations [14] and [6]. Consider a case with $W_f/W = 0.2$, $W_l/W = 0.8$, which requires a whole body solubility coefficient $\alpha' = 2.52$ by Equation [12]. An uncertainty of $\pm$ 15 per cent is assigned to this number, giving $\alpha' = 2.52 \pm 0.378$. This uncertainty, due essentially to uncertainty in q_∞ (see Equation [13]), was obtained by tripling the partial uncertainty in q_∞ estimated in curve-fitting the experimental q(t) data by a sum of exponential functions (28). The preceding numbers and those for α_f', α_l' yield, in Equation [14],

$$\frac{W_f}{W} = \frac{(2.52 \pm 0.378) - (0.33 \pm 0.073)}{(11.26 \pm 0.40) - (0.33 \pm 0.073)} = \frac{2.19 \pm 0.385}{10.93 \pm 0.407} = \frac{2.19\,(1 \pm 0.176)}{10.93\,(1 \pm 0.0372)} = 0.20\,(1 \pm 0.180) = 0.20 \pm 0.036 \quad [20]$$

and, by Equation [6]

$$\frac{W_l}{W} = 1 - \frac{W_f}{W} = 1 - 0.20 \pm 0.036 = 0.80\,(1 \pm 0.045). \quad [21]$$

Thus, for a normal subject the cyclopropane uptake method in its present state of development gives an estimated uncertainty of $\pm$ 18 per cent for total body fat and $\pm$ 5 per cent for fat-free lean mass.

The primary source of uncertainty in the cyclopropane uptake method is the uncertainty of 15 per cent assigned to α' and due essentially to a like uncertainty in q_∞. If this uncertainty can be reduced to 5 per cent, as is thought possible, then the uncertainty in the body fat value would be reduced to about 8 per cent and in fat-free lean mass value to about 2 per cent. This accuracy would be achieved despite an uncertainty of about 3.5 per cent in α_f' and of the large uncertainty of about 21 per cent in α_l'. The latter uncertainty has little influence because α_l' is quite small relative to α_f'.

More generally, the fractional error in W_f is obtained from Equation [14] by differentiation and root mean square addition of random errors as

$$\frac{\delta W_f}{W_f} = \left[\frac{(\delta\alpha')^2 + (\delta\alpha_l')^2}{(\alpha' - \alpha_l')^2} + \frac{(\delta\alpha_f')^2 + (\delta\alpha_l')^2}{(\alpha_f' - \alpha_l')^2} \right]^{\frac{1}{2}} \quad [22]$$

and by Equation [6],

$$\frac{\delta W_l}{W_l} = \frac{W_f}{W_l}\frac{\delta W_f}{W_f} \quad [23]$$

where $\delta\alpha'$ is the uncertainty in α', and so forth. Equation [22] indicates that unless total body fat is less than about 5 per cent of body weight, the main error in the total body fat is due to uncertainty in α', i.e., uncertainty in q_∞. Below this range, error in α_l' becomes important.

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VITAMIN D-RESISTANT RICKETS: THE EFFECT OF CALCIUM INFUSION ON PHOSPHATE REABSORPTION *

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Vitamin D-resistant rickets (R.R.) is a genetically transmitted form of rickets that fails to respond to treatment with nutritional doses of vitamin D. It is characterized by a low serum phosphorus, a normal serum calcium, and an elevated serum alkaline phosphatase, and is transmitted by a sex-linked dominant inheritance pattern (2).

In the first detailed study of R.R., Albright, Butler and Bloomberg (3) demonstrated poor intestinal absorption of calcium and hyperphosphaturia. Nutritional doses of vitamin D had no effect on these metabolic abnormalities, but massive doses improved calcium absorption and decreased the hyperphosphaturia.[1] Albright and colleagues therefore suggested that the poor absorption of calcium was a stimulus for increased parathyroid hormone secretion, which in turn caused the hyperphosphaturia.

In recent years, many studies of R.R. have demonstrated that the fraction of filtered phosphate reabsorbed (TRP) is low despite the presence of a low serum phosphorus concentration. Considering this finding and the similarity of R.R. to the Fanconi syndrome and renal glycosuria, Dent and Harris (4, 5), Fanconi (6) and others have concluded that R.R. is caused by an isolated renal tubular defect of phosphate reabsorption. This opinion has been widely accepted and has led to general usage of the term "phosphate diabetes" as a synonym for the disease.

The present studies were performed to distinguish between a primary tubular defect and extra-renal causes for the hyperphosphaturia in R.R. If the hypothesis of Albright is correct, an elevation of the serum calcium by the infusion of calcium salts should abolish the secondary hyperparathyroidism and, consequently, increase the phosphate reabsorption. On the other hand, if an absolute intrinsic defect of renal phosphate transport exists, the calcium infusion should have little effect on phosphate reabsorption.

The infusion of calcium in R.R. was associated with a definite increase of phosphate reabsorption. This evidence, combined with appropriate control studies, indicated that extra-renal factors are in part responsible for the characteristic pattern of phosphate reabsorption.

MATERIAL AND METHODS

Eight patients, 7 females and 1 male, with the characteristic features of R.R. were studied. They were derived from 3 families belonging to a group of patients under observation at the Shriners Hospital of St. Louis (7). The patients selected for study had received no vitamin D therapy for at least 3 years and had not had any orthopedic operations resulting in immobilization for at least 1 year before the study. The age of the patients ranged between 7 and 53 years. Physical and laboratory examinations were done to exclude acidosis, glycosuria, aminoaciduria, overt renal impairment, infection, or other intercurrent illnesses.

Ten control subjects were studied. These included 2 normal medical students, 4 normal siblings of patients with R.R., and 4 patients on the general medical wards of Barnes Hospital who were under observation for diseases unrelated to skeletal or metabolic disease and did not receive treatment during the study. The results obtained in the 4 siblings were indistinguishable from those in other normal control subjects and were therefore included in the control group. A patient with postoperative hypoparathyroidism was also studied.

Phosphate and creatinine clearances were measured simultaneously in the postabsorptive state. Patients were given 1 L of water orally at the beginning of each study

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[1] The urinary phosphate excretion may not be increased in absolute quantities. However, it is always greater than normal considering the low serum phosphorus concentration. In the absence of hyperparathyroidism or a renal tubular defect, a low serum phosphorus concentration is associated with a very low urinary phosphate excretion.

and additional water throughout the study in order to assure adequate urine flows. Clearance periods varied from 2 to 4 hours, and 5 to 6 clearance periods were obtained in each study. Urine was collected by voiding. Samples of venous blood were obtained at the mid-point of each period. Phosphorus was determined by the method of Gomori (8) and creatinine by the method of Folin as modified by Hawk, Oser and Summerson (9). In 50 normal adult subjects, the creatinine clearance measured with this method averaged 95 ml per minute (range, 70 to 110). Calcium was determined by flame photometry (Coleman flame photometer, model 21). The results are expressed as amount of phosphate reabsorbed per minute and as per cent TRP. It is assumed that all of the inorganic serum phosphate is ultrafiltrable and that the excreted phosphate represents the portion of filtered phosphate which is not reabsorbed. Phosphate secretion by the tubules has been demonstrated in the chicken (10) and has been suggested by recent studies in the dog (11, 12). To our knowledge, however, there is no convincing evidence of tubular phosphate secretion in man. Since the clearances of phosphate and creatinine were determined from measurements performed on the same samples of blood and urine, it should be noted here that errors due to incomplete bladder emptying did not affect the calculated value of the TRP.

For at least 3 days before an infusion, the subjects were given a hospital diet supplemented by a minimum of 1 quart of milk daily in order to assure an adequate phosphate intake. After an overnight fast with an unrestricted intake of water, 1 or 2 control clearance periods were obtained. An infusion of 15 mg of calcium per kg of body weight as the gluconate-glucoheptonate (Abbot) in saline was then given intravenously at a

TABLE I

Patterns of renal excretion of phosphate before, during and after infusion of calcium *

Age	Period	Creatinine clearance	Serum P	Filtered P	Excreted P	Reabsorbed P	TRP	Serum Ca
yrs		*ml/min*†	*mg/100 ml*		*μmoles/min*		*%*	*mg/100 ml*
				A. Normal subjects				
33	A	67.1	4.90	106	20.4	85.6	80.9	10.1
	B	66.7	4.82	104	14.2	89.8	86.3	11.4
	C	68.3	5.37	118	15.6	102	86.4	12.3
	D	67.6	5.84	127	15.6	111	87.4	12.7
	E	68.1	6.23	137	19.6	117	85.4	11.7
52	A	90.4	3.59	104	9.5	94.5	90.9	9.5
	B	123	3.91	155	14.4	141	91.0	10.4
	C	106	4.39	150	16.1	134	89.3	12.2
	D	80.4	4.77	124	13.2	111	89.4	12.0
	E	70.2	5.16	117	8.5	108	92.3	11.4
				B. Rachitic subjects				
7	A	66.8	1.86	40.1	26.5	13.6	33.9	10.2
	B	77.1	1.94	48.3	16.2	32.1	66.4	11.2
	C	88.8	2.17	62.2	26.9	35.3	56.7	12.7
	D	81.5	2.55	67.0	10.7	56.3	84.0	10.7
	E	77.5	2.55	63.8	17.4	46.4	72.7	10.8
16	A	106	2.54	86.8	16.9	69.9	80.5	9.9
	B	108	2.67	93.0	19.0	74.0	79.6	11.1
	C	133	2.98	128	15.1	113	88.3	12.0
	D	106	3.03	104	10.3	93.7	90.1	11.7
	E	137	3.01	133	11.0	122	91.7	9.9
18	A	120	2.11	81.7	19.3	62.4	76.4	9.6
	B	120	2.50	96.8	17.8	79.0	81.6	11.8
	C	115	2.73	101	13.2	87.8	86.9	12.4
	D	105	2.78	94.2	5.8	88.4	93.8	11.6
	E	96.5	2.51	78.1	6.1	72.0	92.2	10.1
53	A	106	1.92	65.7	10.0	55.7	84.8	9.7
	B	97.6	2.06	64.8	5.7	59.1	91.2	10.6
	C	108	2.98	104	7.6	96.4	92.7	11.6
	D	147	3.55	168	10.8	157.2	93.6	11.9
	E	118	3.51	134	4.9	129	96.3	11.2

* Period A, before infusion (average of two 2-hour or one 4-hour clearance); Periods B and C, during infusion (two consecutive 2-hour clearances); Periods D and E, after infusion (two consecutive 2-hour clearances). P = phosphorus; TRP = tubular reabsorption of phosphate; Ca = calcium.

† Corrected to 1.73 m^2 body surface.

constant rate over 4 hours. Two clearance periods of 2 hours each were obtained during the infusion and 2 immediately following the infusion.

RESULTS

Normal subjects. The mean response of the TRP during the calcium infusion studies in 10 subjects is shown in Figure 1, and details of two representative studies are shown in Table I, *A*. On a random diet, the baseline TRP in normal subjects in this laboratory is 85 ± 3 per cent, with 95 per cent confidence limits of 77 to 93 per cent (13). The serum phosphorus increased during the infusion in every case, in agreement with previously reported data (14). The creatinine clearance remained constant or increased. As a result of these changes, the filtered load of phosphate always increased. In general, the reabsorbed phosphate increased in proportion to the filtered phosphate so that the TRP rose only slightly (Figure 1). During and following the infusion, the phosphate reabsorption was often very high and in the range of values of maximal tubular phosphate reabsorption reported by Thompson and Hiatt [(15), Table I, *A*].

Rachitic subjects. The mean response of the TRP during the calcium infusion studies in the eight rachitic subjects is shown in Figure 2, and details of four representative studies are shown in Table I, *B*. The serum phosphorus always increased during the infusion, as it did in normal subjects, and changes in the creatinine clearance were variable with a tendency toward an increase during the infusion. Both filtered and reabsorbed phosphate always increased. However, the reabsorbed phosphate increased more than the filtered so that the fraction of TRP increased markedly.

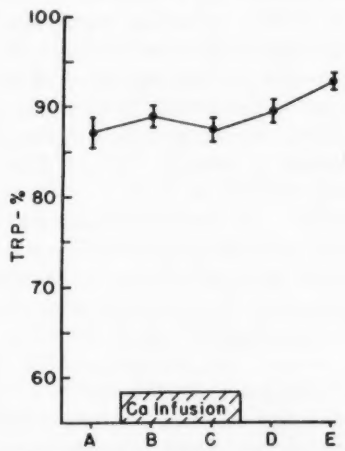

FIG. 1. EFFECT OF CALCIUM INFUSION ON FILTERED PHOSPHATE REABSORBED (TRP) IN TEN NORMAL SUBJECTS. Mean ± 1 SE. A was a 4 hour control period; all other clearance periods extended over 2 hours; 15 mg of calcium per kg of body weight was infused at a constant rate over 4 hours.

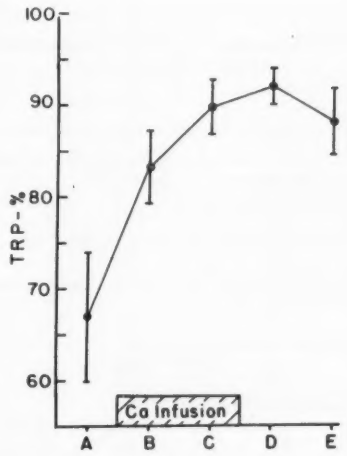

FIG. 2. EFFECT OF CALCIUM INFUSION ON TRP IN EIGHT RACHITIC SUBJECTS. Mean ± 1 SE. A was a 4-hour control period; all other clearance periods extended over 2 hours; 15 mg of calcium per kg of body weight was infused at a constant rate over 4 hours.

Hypoparathyroid patient. In order to test the possibility that a calcium infusion may alter renal phosphate transport independently of changes in parathyroid activity, the effects of a calcium infusion were studied in a hypoparathyroid subject who was maintained on constant doses of parathyroid extract (Table II). The patient developed hypoparathyroidism after a thyroidectomy three years previously and had not received vitamin D for four months before the study. Parathyroid extract (Lilly) was administered for the three day period preceding the study. On the day of the calcium infusion study, parathyroid extract was given intravenously at a constant rate.

Administration of parathyroid extract resulted in a striking increase of the creatinine clearance. However, owing to the concurrent decrease of the serum phosphorus concentration from 5.8 to 2.6 mg per 100 ml, the filtered phosphate decreased

TABLE II

Pattern of renal excretion of phosphate in a hypoparathyroid subject *

Creatinine clearance	Serum P	Filtered P	Excreted P	Reabsorbed P	TRP
ml/min†	*mg/100 ml*		*μmoles/min*		%
Baseline					
57.0	5.81	107	5.5	103	96.2
After 3 days' i.m. parathyroid extract, 200 U q6hr					
118	2.59	98.6	20.9	77.7	78.8
2 hrs after i.v. parathyroid extract, 50 U/hr, maintained throughout remainder of experiment					
106	2.58	88.2	24.4	63.8	72.3
During calcium infusion, 15 mg/kg					
115	2.86	106	28.4	77.6	73.2
117	2.99	113	37.0	76.0	67.2
Following calcium infusion					
112	3.27	118	41.4	76.9	65.2
109	3.52	124	40.7	83.3	67.2

* P = phosphorus; TRP = tubular reabsorption of phosphate. The first two clearance periods lasted 4 hours, the others 2 hours.
† Corrected to 1.73 m^2 body surface.

slightly (from 107 to 99 μmoles per minute). The reabsorbed phosphate fell from 103 to 78 μmoles per minute while a 4.6-fold increase of phosphate excretion occurred. A transitory decrease of filtered as well as reabsorbed phosphate occurred 2 hours after beginning the intravenous infusion of parathyroid extract. During and following the infusion of calcium, the filtered phosphate increased, chiefly as a result of an increase of the serum phosphorus concentration, while the reabsorbed remained nearly constant at approximately 77 μmoles per minute. The TRP consequently decreased. This response should be contrasted with that of patients whose parathyroid glands are intact: in the presence of the glands, the invariable increase of the filtered phosphate induced by the calcium infusion was associated with a disproportionately large increase of the reabsorbed phosphate and therefore an increase of the TRP.

DISCUSSION

The literature contains brief references to the use of intravenous infusions of calcium in the differentiation between intrinsic renal and extrarenal causes of impaired phosphate reabsorption in R.R. Lamy, Royer, Frézal and Lestradet (16) noted a decrease in phosphaturia and an increase of the TRP from 82 to 98 per cent during a calcium infusion in a patient with typical R.R. Lestradet, Royer and Jacob (17) studied five patients with R.R. before and following an infusion of calcium. A moderate increase of the TRP was noted in three patients and a decrease in the two other patients. However, this study was not comparable with the present one because the calcium was administered in a solution containing 4 per cent glucose; the infusion of glucose was likely to be associated with a decrease of the serum phosphorus concentration. Data on changes in serum phosphorus concentration and details of phosphate excretion were not given. In their review of the literature on R.R., Winters, Graham, Williams, McFalls and Burnett (2) refer to unpublished observations on the effects of a calcium infusion in two patients with R.R. The phosphaturia was diminished, but "phosphorus did not completely disappear from the urine as would have been expected in the presence of marked hypophosphatemia, if the resorptive mechanism had been converted to complete normality. . . ." Fraser, Leeming, Cerwenka and Kenyeres (18) published preliminary observations on two patients with R. R. with calcium infusions and noted rapid diminution of the excessive urinary output of phosphorus throughout the period of calcium infusion.

In the present studies, the infusion of calcium was associated with a striking increase of the rate of phosphate reabsorption in every case. This observation indicates that extra-renal factors are of major importance in causing the abnormal pattern of phosphate excretion in R.R. It is not possible to state whether phosphate reabsorption was completely restored to "normal" by the calcium infusion, because phosphate reabsorption is highly variable in normal subjects. This is true both under baseline conditions and during phosphate loading. The maximal tubular reabsorptive rate often does not attain a consistent value in subjects studied repeatedly (15). Although parathyroid extract is known to decrease the Tm_p, there is a considerable overlap of values of the Tm_p in normal and hypoparathyroid subjects (15, 19). During and following the calcium infusion, phosphate reabsorption in the rachitic subjects often attained values within the range of the normal Tm_p, even though the plasma concentration of phosphorus was far below that which is commonly reached in studies of the Tm_p, and full saturation of the transport system for phosphate would therefore not be expected.

In recent years, the TRP has been widely used as an indirect measure of parathyroid function. The TRP is low in hyperparathyroidism and high in hypoparathyroidism (*20*). It is not, however, a specific measure of parathyroid function. The TRP is not a discrete measure of phosphate transport but a ratio relating tubular reabsorption of phosphate to the filtered load of phosphate. This seems to be an appropriate expression for the following reasons. *1*) Comparison between normal and rachitic subjects is facilitated by factoring reabsorbed phosphate by the filtered load; the filtered load decidedly affects reabsorption and is always low in the rachitic subjects owing to the characteristic hypophosphatemia. *2*) It was of interest to explore the possibility that changes in phosphate reabsorption during the calcium infusion might be related to changes in parathyroid function. Crawford, Osborne, Talbot, Terry and Morrill (*21*) have shown that the TRP is a more sensitive index of parathyroid function than the absolute rate of phosphate reabsorption: the TRP has an essentially constant high value in parathyroidectomized animals and promptly decreases when parathyroid extract is administered. The impressive increase of the TRP during and following the calcium infusions may therefore be related to decreasing parathyroid function. While this conclusion is open to doubt in the absence of more direct measures of parathyroid function, it is consistent with the available data. The control observation in the subjects with hypoparathyroidism receiving a constant amount of parathyroid extract lends support to this explanation by showing that the calcium infusion does not induce an increase of the TRP when fluctuations of parathyroid function are not possible. The small decrease of the TRP actually observed in this experiment is consistent with the increase of filtered phosphate, which may by itself cause a slight depression of the TRP in the hypoparathyroid state (*22*).

The present experiments do not shed any light on the important observation that there are carriers of R.R. who have hypophosphatemia without overt bone disease (*2*, 23). Active bone disease is believed to develop because the concentration of serum phosphorus is so low as to prevent normal calcification of osteoid. It is known that sera obtained from rachitic patients fail to effect normal calcification of rachitic rat cartilage *in vitro* (*24*, *25*) and that rapid calcification can be achieved in rachitic subjects by the continued infusion of phosphate and calcium (*25*). These observations focus attention on the hypophosphatemia as the immediate precursor of the bone disease and appear to show that an intrinsic defect of bone does not exist. The existence of asymptomatic carriers of the disease represents a paradox that holds the key to a more comprehensive explanation of the etiology of R.R.

SUMMARY

1. The effects of the intravenous infusion of calcium were studied in patients with vitamin D-resistant rickets and in control subjects. The infusion of calcium consistently increased the low tubular reabsorption of phosphate of resistant rickets to nearly normal levels.

2. The calcium infusions were always accompanied by an increase in the serum phosphorus, the filtered phosphate, and the reabsorbed phosphate. This is interpreted as suggesting decreased parathyroid function.

3. It is concluded that secondary hyperparathyroidism, presumably due to defective gastrointestinal absorption of calcium, accounts, at least in part, for the phosphaturia and the hypophosphatemia characteristic of the disease.

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SUSCEPTIBILITY TO EXPERIMENTAL PYELONEPHRITIS WHEN HORMONAL HYPERTENSION IS PREVENTED BY HYPOTENSIVE DRUGS *

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Rats with desoxycorticosterone (DCA)-saline induced hypertension have been shown to exhibit increased susceptibility to experimental pyelonephritis (1, 2). These studies lend support to the hypothesis that various injuries, including nephrosclerosis, predispose to human pyelonephritis and that pyelonephritis is superimposed upon hypertensive disease more frequently than is usually suspected. Others (3–5) have shown that reserpine and/or hydralazine, when given to rats with DCA-saline hypertension, will reduce blood pressure, delay or prevent renal and vascular pathological changes, and increase the average survival time. Masson, McCormack, Dustan and Corcoran (6) demonstrated a close association between blood pressure levels and the presence and character of the vascular lesions in rats with hydralazine-treated *renal* hypertension. The previously demonstrated susceptibility of rats with DCA-saline hypertension might have been due either to an effect of the hypertension and vascular disease or to some other effect of the administered steroid and saline. The present investigation was therefore designed to separate these possibilities.

MATERIALS AND METHODS

White, female Sprague-Dawley strain rats, weighing 100 to 150 g, were used. The experimental animals of Groups A and B were subjected to left nephrectomy and 3 fortnightly injections of a long-acting derivative of DCA [1] in 25 mg doses. Drinking water contained 1 per cent NaCl and 0.49 per cent KCl. The latter had been previously found (1) to prevent potassium depletion which otherwise results from the DCA-saline regimen. In addition, 40 mg of hydralazine [1] was added to each liter of the drinking solution of Group A animals and they received 1 mg per kg (later reduced to 0.5 mg per kg) of syrosingopine [1] subcutaneously each day. Syrosingopine was chosen rather than reserpine since it has been found to have little, if any, effect on spontaneous salt appetite (7).

The animals of Group C had a left nephrectomy but received no DCA and drank ordinary tap water. All animals ate Purina Laboratory Chow. The animals were "pair-fed" by groups, i.e., the intakes of Groups A and C were limited each day to the average intake of Group B. Additionally, the fluid intake of Group B animals was limited each day to the average fluid intake of Group A in order to adjust for any effect syrosingopine might have on spontaneous salt intake. The resulting weight curves of the three groups were very similar, as is shown in Figure 1.

The method of determining blood pressure by use of a microphonic manometer and the bacteriological techniques employed have been previously described (1, 8). Rats of the three groups designated for bacterial injection received an inoculum of 100,000,000 *Escherichia coli* i.v.

Experimental design. The experimental design is shown in Table I. Administration of hypotensive drugs and the DCA-saline regimen were started simultaneously.

Intravenous injection of *E. coli* was carried out 7 weeks later and the rats sacrificed 2 weeks following injection. Six rats of Groups A and B and 5 of Group C did not receive *E. coli*, in order to facilitate histological study of

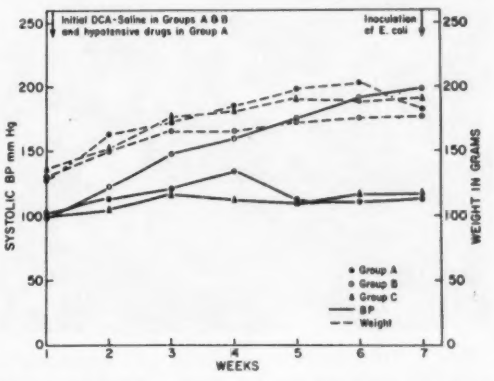

FIG. 1. AVERAGE WEIGHTS AND SYSTOLIC BLOOD PRESSURES OF GROUPS A, B AND C.

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[1] Kindly supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

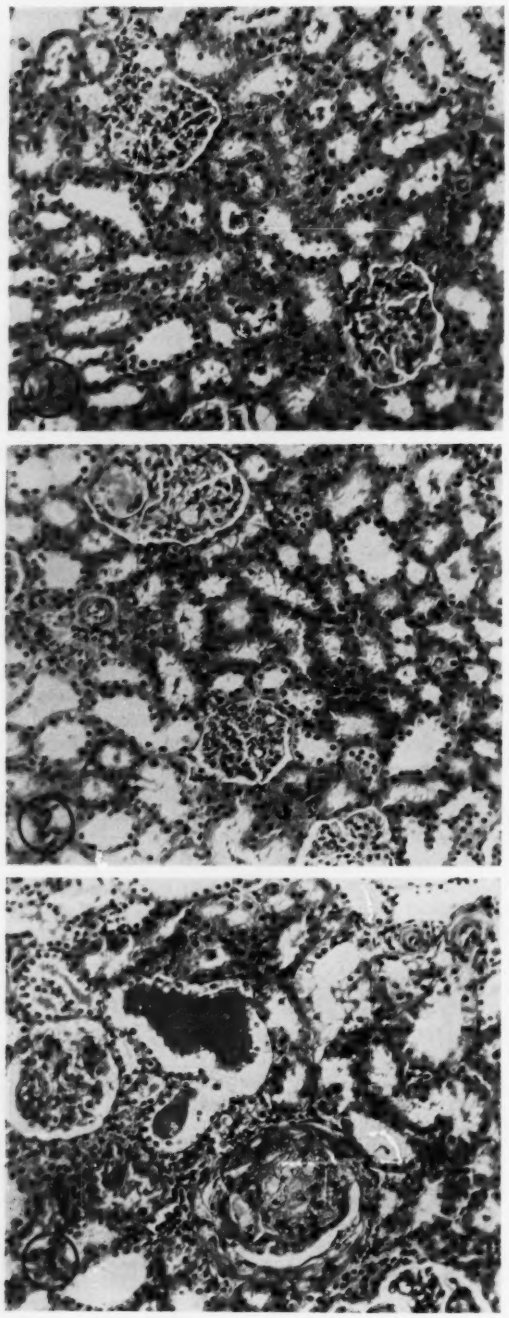

FIG. 2. ILLUSTRATIONS OF NORMAL AND PATHOLOGICAL HISTOLOGY ENCOUNTERED. 1. Kidney of control rat (previously uninephrectomized). 2. Kidney from an uninjected rat of Group A showing a nodular deposit of material with the staining characteristics of fibrinoid in a glomerulus and very slight tubular dilatation. 3. Kidney from an uninjected rat of Group B showing periglomerular fibrosis, glomerular sclerosis, marked dilatation of tubules, some of which contain casts, and minimal arteriolar sclerosis. Hematoxylin and eosin (× 200).

TABLE I

Experimental design

Group	Description	No. of rats receiving *E. coli* i.v.	No. of rats uninjected with *E. coli*
A	DCA-saline regimen uninephrectomy syrosingopine-hydralazine	15	6
B	DCA-saline regimen uninephrectomy no hypotensive drugs	16	6
C	Uninephrectomized controls	14	5

the kidneys of these representatives after sacrifice. At the time of sacrifice, the remaining right kidney of each animal was removed with aseptic technique, divided in half longitudinally, and one-half cultured quantitatively while the other half was fixed in 10 per cent formalin for histological sections.

RESULTS

General condition of animals. While equal numbers (nine) of rats died in Groups A and B *prior* to injection of *E. coli* there appeared to be a difference between the two groups. All of the Group A rats were markedly edematous at the time of death and six of nine died within the first eight days of the experiment. This, correctly or not, was attributed to the syrosingopine, and the mortality rate and development of edema seemed to decline markedly when the dose of this drug was reduced from 1 to 0.5 mg per kg per day. Deaths of the nine rats of Group B were fairly evenly distributed over the interval of 6 to 20 days after onset of the experiment and all but one were thin and sickly in appearance. The one exception was edematous. Four of the control animals in Group C also died—one accidentally, one from intestinal obstruction, and two from unknown cause. The remaining animals of the three groups maintained body weight, appeared reasonably healthy, and all survived to the time of sacrifice, two weeks following intravenous injection of *E. coli.*

Systolic blood pressure. As is seen in Figure 1, the combination of syrosingopine and hydralazine was highly successful in preventing severe hyper-

tension. The average of the blood pressures of the Group A rats was practically identical with that of the controls of Group C at the time of bacterial injection. There were individual variations in blood pressure levels, but no animal in this group had a reading above 160 mm Hg and most had pressures below 130 mm during the greater part of the seven week period. The average blood pressure for Group B, on the other hand, rose progressively to 200 mm Hg by the end of seven weeks. The highest pressure recorded in this group was 236 mm Hg.

Renal histology. The rapid development of hypertension in the albino rat as a result of the DCA-saline regimen is usually accompanied by the appearance of widespread acute vascular disease (5, 9). Arteriolar sclerosis is present and some arterioles contain subendothelial material with the characteristics of fibrinoid. Glomeruli show enlargement and capsular fibrosis. Convoluted tubules are often dilated and contain eosinophilic colloid. The above described lesions were present to a moderate or severe degree in the kidneys of rats of Group B. In most of the rats of Group A, there were no or minimal glomerular and tubular changes, and fibrinoid arteriolar lesions were rare. The two instances in this group of more advanced renal changes also had the highest systolic blood pressure recorded. Illustrations of kidneys of normal (uninephrectomized) rats, and of those of Groups A and B are shown in Figure 2.

Susceptibility to experimental pyelonephritis. The results are shown in Table II. Of the 15 rats of Group A receiving one hundred million *E. coli* intravenously, 4 had unequivocal pyelonephritis. One had no histological evidence of infection, a colony count of only 800 bacteria per ml of renal tissue, and was considered uninfected. Of the 16 rats of Group B, 10 had clear-cut evidence of pyelonephritis. One animal had both gross and microscopic evidence of abscess formation in the half-kidney which was fixed for sections, while the remaining half had no gross abscesses and was sterile on culture. The null-hypothesis stating that the incidence of infection is the same for rats in Groups A and B was rejected in favor of the alternative hypothesis that if a difference did in fact exist, it would be in the direction of rats in Group B having a greater incidence of infection than rats in Group A. This conclusion was arrived at after application of the appropriate *one-tailed test* of significance based upon Fisher's exact method for a 2×2 contingency table (10). The calculated probability of an error of the first kind (viz., that of falsely concluding that the incidence of infection for rats in Group B is greater than that for rats in Group A) is $p = 0.049$. Only one control of Group C had renal infection. This was evidenced by a microscopic abscess and a colony count of 9,500 organisms per ml of kidney. The difference in incidence of infection between Groups B and C is highly significant ($p = 0.002$), as was

TABLE II

Colony counts and descriptions of kidneys of the three groups two weeks after injection of E. coli

Group A		Group B		Group C	
Colony counts	Description*	Colony counts	Description	Colony counts	Description
0	0	1,200,000	++	0	0
3,700,000	+++	8,000,000	+++	0	0
800	0	11,000,000	+++	0	0
5,000,000	+++	2,000,000	+++	0	0
20,000,000	+++	2,100,000	++	0	0
2,000,000	++	0	+++	0	0
0	0	100,000	++	0	0
0	0	1,400,000	+++	0	0
0	0	18,000,000	++	0	0
0	0	0	0	0	0
0	0	0	0	9,500	++
0	0	100,000,000	+++	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0		
		0	0		

* 0, no cellular infiltrate; ++, microscopic abscesses; +++, macroscopic abscesses.

previously found (1). There was no significant difference in incidence of infection between Groups A and C.

DISCUSSION

The renewal of interest in and great increase in research on pyelonephritis and unilateral renal disease associated with hypertension in recent years have raised many questions regarding the interrelationships of these diseases in man. Few would question that chronic bilateral pyelonephritis may produce severe hypertensive disease, but it often does not, even when leading to uremia. Much skepticism has arisen regarding the role of unilateral pyelonephritis and hypertension, since removal of the affected kidney so rarely results in alleviation of the hypertension. Even the production of hypertension in the experimental animal by means of pyelonephritis has not been firmly established. If such an experimental model can be developed, it should be quite helpful in the elucidation of these problems.

The reverse relationship, i.e., that hypertensive renal disease may predispose to pyelonephritis, is only suggested so far by a few human and animal investigations (1, 2, 11, 12). Goldblatt (13) has hypothesized that malignant hypertension may develop from benign hypertension because of the superimposition of renal infection. Long-term studies of human normal and hypertensive populations will be required to settle these questions.

The data reported here again show that hypertensive disease induced by DCA and saline leads to greatly increased susceptibility to experimental pyelonephritis. They also suggest that the blood pressure itself is an important factor in the production of renal damage and heightened susceptibility, since when it is controlled at normal or near normal levels, renal arterial and arteriolar lesions are minimal or absent and susceptibility to infection is reduced. That the blood pressure may not be the only factor is demonstrated by the recent experiments of Gardner (5). He showed that, in rats with steroid hypertension receiving daily intramuscular injections of hydralazine, acute vascular disease can be prevented despite discontinuous reduction in the mean systemic blood pressure and suggested that this prevention was due either to the discontinuous effect on blood pressure or to "a factor, at present unrecognized, which is more sensitive to the action of the drug than is the blood pressure." In this study, hydralazine was present in the drinking solution, syrosingopine was administered additionally, and a continuous hypotensive effect is assumed. However, it cannot be proved, since blood pressure measurements were made at weekly intervals.

This study has made no attempt to investigate the effect of hypotensive drugs on survival time of the animals and, while the doses of syrosingopine used during the first week of the experiment in Group A seemed to cause edema and early death, this is not certain. In this investigation, there was no significant difference in mortality rate during the seven week period prior to bacterial injection between the rats with controlled and untreated hormonal hypertension.

SUMMARY

Rats with desoxycorticosterone-saline induced hypertension have again been found to exhibit marked renal vascular damage and increased susceptibility to experimental pyelonephritis. In other rats on the same regimen to which hypotensive drugs were added, blood pressure was at or near normal levels when measured at weekly intervals, renal vascular lesions were minimized, and no increased susceptibility to pyelonephritis was demonstrated.

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OXIDATIVE HEMOLYSIS AND PRECIPITATION OF HEMOGLOBIN. I. HEINZ BODY ANEMIAS AS AN ACCELERATION OF RED CELL AGING *

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The propensity of certain chemicals and drugs to induce hemolytic anemias has been a subject of much interest for almost a century (1–4). The majority of substances so active are aromatic compounds containing amino-, nitro-, or hydroxy groups, although a number of inorganic compounds (e.g., hydroxylamines, nitrates, nitrites and chlorates) are also active (1–4). Such compounds usually cause red cell injury without evidence of specific toxicity to other cells or tissues. The hemolytic process is characterized by two rather distinctive features in the affected red cells: *1*) the appearance of brownish or greenish derivatives of hemoglobin, including methemoglobin, according to most (3) but not all (4) reports; and *2*) the formation within red cells of water-insoluble, stainable granules, generally termed Heinz bodies.

There has been extensive debate as to the identity and significance of the hemoglobin discoloration following the administration of these compounds. Much of the disagreement may be attributed to the fact that many of the observations have been made *in vivo,* where pigments initially produced by the compound are mixed with the various products of hemoglobin catabolism. Furthermore, the fact that certain of the pigments produced, such as methemoglobin, are reversible, whereas certain others (including so-called sulfhemoglobin) are not (3), has undoubtedly led to conflicting observations. In those few studies where early, sequential observations have been made, methemoglobinemia has been striking albeit transient (5, 6). Studies of compounds such as phenylhydrazine, which are active against red cells *in vitro* as well as *in vivo,* have shown that hemoglobin may be changed in part to methemoglobin and in part to brown or green pigments, some of which are insoluble (7, 8), and which include the hemochromes, sulfhemoglobin (3, 7), verdoglobin (7, 9, 10), and choleglobin (3, 11). These pigments are not well defined chemically and may be considered collectively as various forms of irreversibly denatured hemoglobin with heightened absorption of red light.

An even more voluminous and conflicting body of literature has arisen concerning the nature of the Heinz body (1, 2). The most prevalent of many views has been that Heinz bodies consist, at least in part, of particles of denatured protein (1) and these are presumed to include denatured globin (2). Indeed, denatured globin has been identified in the blood of animals with Heinz body anemias (10, 12) and Heinz bodies have been found to have the staining properties of globin rather than of stromal protein (8). However, studies by electron microscopy were interpreted by one observer to indicate that these inclusions were derived from denatured stromal protein (13), while others concluded that the particles derived from affected blood were too opaque to be denatured protein (14).

A third less specific and possibly less constant feature of the Heinz body anemias is the appearance of spherocytosis and increased osmotic fragility (15, 16). As with the methemoglobinemia, conflicting reports as to the occurrence or absence of spherocytosis may be in part attributed to mechanisms *in vivo,* such as splenic filtration, which would tend to eliminate spherocytes from the blood stream. Many of these compounds which produce methemoglobin and Heinz bodies *in vitro* also cause spherocytosis and increased osmotic fragility *in vitro,* which usually become apparent only after several hours (15, 17).

The injurious effects of the hemolytic drugs *in vivo* and *in vitro* are modified by the character

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of the red cells involved. Thus, red cells of some species, such as the cat, are more susceptible to injury in terms of methemoglobin formation as well as of hemolysis *in vivo* and *in vitro* (17) than those of other species. Old red cells are more susceptible than young (16, 18). The red cells of some individuals, particularly certain Negroes and dark-skinned Caucasians (19), are more susceptible than those of most individuals. The mechanism of individual hypersusceptibility [1] has been well characterized in recent years (19), and apparently depends upon an inborn, genetically-determined metabolic error resulting in a deficiency of the enzyme glucose-6-phosphate dehydrogenase (20). This enzyme catalyzes the first step of the pentose phosphate pathway which provides the mature red cell with its only oxidative apparatus and which is responsible for the generation of reduced triphosphopyridine nucleotide (TPNH). TPNH, in turn, is possibly involved along with DPNH (21) in the enzymatic reduction of methemoglobin (22) and of oxidized glutathione (GSSG) (23, 24). Since reduced glutathione (GSH) levels in the red cells of hypersusceptible individuals tend to be low and to fall more rapidly than normal in the presence of the hemolytic compounds (19), and since glucose-deficient normal red cells are also "hypersusceptible" (19), it is generally accepted that oxidative glycolysis provides a cellular defense against these drugs, possibly through the preservation or regeneration of GSH. Normal red cells become hypersusceptible in the same sense as they grow old, for they too are deficient in glucose-6-phosphate dehydrogenase (25) and thereby manifest "glutathione instability" (19).

Whether or not GSH per se is protective to the drug-exposed red cell, and, if so, in what way, is not clear. GSH levels and "GSH stability tests" on the red cells of hypersusceptible individuals do not appear to correlate with the marked differences in susceptibility occurring before and after drug exposure (26). Furthermore, the fate of GSH in drug-exposed red cells is unknown; it is clear from inspection of the *in vitro* data of Beutler, Robson and Buttenwieser (27) and from *in vivo* studies (26), that GSH is not simply oxidized to GSSG. Finally, there are certain unexplained differences after drug exposure in the pattern of hemolysis and in the appearance of the Heinz bodies in the hypersusceptible individual as compared to the normal.

The following studies, presented in preliminary form elsewhere (28), were made in an effort to understand the actual mechanism by which the hemolytic compounds cited cause cellular injury and to explain some of the manifestations of this injury. The present report is concerned with the sequence of changes during the degradation of hemoglobin by these compounds, their mechanism of action, and the relation of Heinz body anemias to normal cellular aging. A second paper will present studies of the relation of thiols to the destruction of hemoglobin and of red cells by these hemolytic agents.

MATERIALS AND METHODS

Red cells derived from fresh defibrinated human blood were washed three times in 10 to 20 volumes of cold physiological ("isotonic") saline and then prepared as 50 per cent suspensions in isotonic phosphate buffer, pH 7.4. Human hemoglobin for most studies was prepared from red cells washed five times at 0° C in isotonic saline and hemolyzed at 0° C with 1.5 volumes of water and 0.4 volume of toluene. Subsequently sufficient sodium chloride was added to bring its concentration to 0.16 M and the hemolysate was centrifuged at 0° C for 1 hour at about 1,000 G. The clear hemolysate was withdrawn and dialyzed for approximately 48 hours at 4° C against 2.8 M phosphate buffer, pH 6.8, according to the procedure of Drabkin (29) for preparing crystalline hemoglobin. The crystals were removed by filtration, dissolved as approximately 20 per cent of hemoglobin solution in cold water, and dialyzed for 24 hours against isotonic phosphate buffer, pH 7.4. Immediately before use this freshly dialyzed hemoglobin was spun at 105,000 G for 1 hour at 0° C in a Spinco Model L ultracentrifuge. In some experiments hemoglobin prepared as described above was further purified by column chromatographic separation.[2] For this purpose a column of IRC-50 resin,[3]

[1] The expressions "sensitive" or "insensitive" in regard to the red cells of subjects with glucose-6-phosphate dehydrogenase deficiency, and normal subjects, respectively, may be objected to as suggesting an all-or-none relationship which obscures the fact that all individuals are in some degree susceptible to toxic hemolysis by this family of drugs and chemicals.

[2] Although the procedure of Drabkin is useful for securing isolated crystals of hemoglobin, it is difficult in bulk procedures to exclude small amounts of other proteins which may also precipitate under the conditions employed.

[3] Rohm and Haas Co., Washington Square, Philadelphia, Pa.

equilibrated with a phosphate buffer (developer no. 2) at 7° C, was employed (30).

Unless otherwise indicated the hemolytic compounds employed were dissolved immediately prior to use in cold isotonic phosphate buffer, pH 7.4, except with phenylhydrazine hydrochloride, to which an equivalent of sodium hydroxide was also added. Phenylhydrazine and acetylphenylhydrazine were selected for use in most of the experiments since they are the most extensively studied of the hemolytic compounds and are active *in vitro* as well as *in vivo*.

Unless otherwise stated, hemoglobin or red cell preparations were incubated in siliconized, 50 ml Erlenmeyer flasks in a Dubnoff metabolic shaker. Experiments were terminated by placing the flasks in an ice bath.

Methemoglobin and so-called sulfhemoglobin were measured by the Evelyn-Malloy method (31). Specimens for this purpose were diluted in ice cold M/15 phosphate buffer, pH 6.6, and then filtered through no. 12 Whatman paper before o tical readings were made.

Spectrophotometric analyses were made with the Beckman model 2400 DU spectrophotometer on 0.01 mM hemoglobin solutions dialyzed at 4° C overnight against M/15 phosphate buffer, pH 6.6. Optical density readings were made at 5 mμ intervals over a range of from 200 to 1,000 mμ.

Red cell suspensions were examined microscopically for inclusion bodies after supravital staining with isotonic aqueous solutions of crystal violet and of methyl violet. Suspensions of precipitated hemoglobin were examined in the same way.

The weight of precipitates derived from drug-treated hemoglobin solutions and from water-hemolyzed red cell suspensions was determined by centrifuging the preparations in cellulose tubes at 105,000 G for 30 minutes at 0° C; the supernatant fluid was then decanted and replaced with water and a second, similar centrifugation was performed. Thereafter the supernatant fluid was removed, and the precipitate was desiccated and weighed

Elution chromatography of hemoglobin solutions (30)

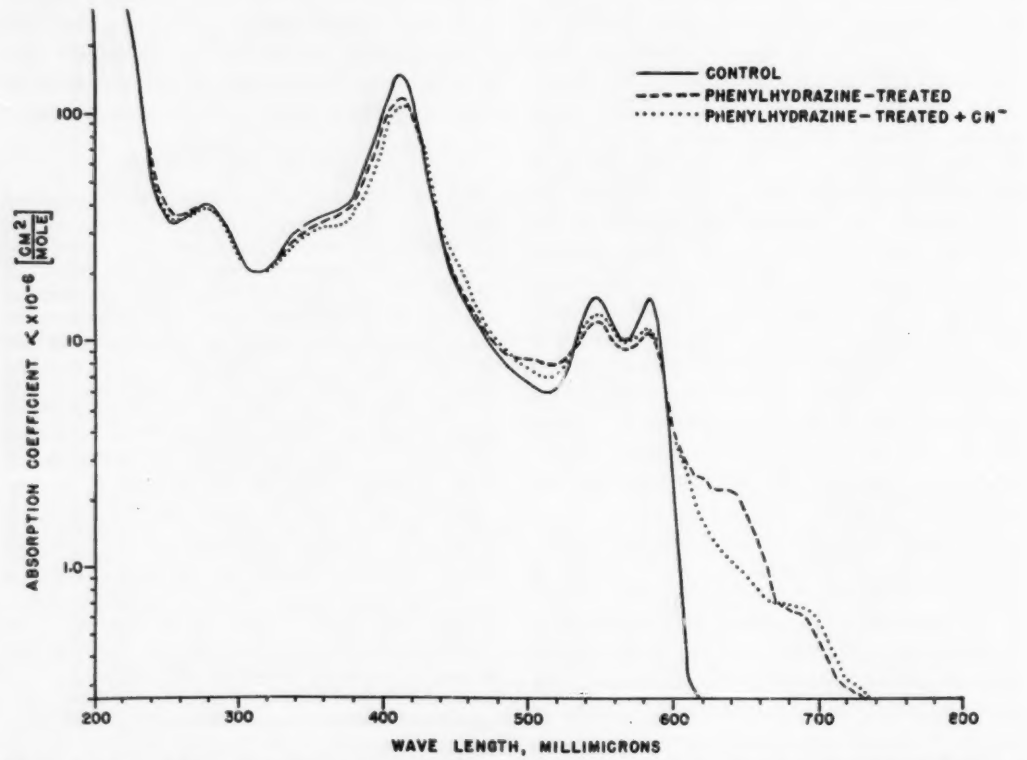


FIG. 1. CHANGES IN LIGHT ABSORPTION CHARACTERISTICS OF HEMOGLOBIN ON EXPOSURE TO PHENYLHYDRAZINE. As compared to the absorption pattern of the "control" hemoglobin (uninterrupted line), hemoglobin exposed to phenylhydrazine (interrupted line) showed a diminution of the Soret peak at 410 to 420 mμ and of the twin peaks at 540 and 580 mμ characteristic of oxyhemoglobin. The increased absorption at 500 and 635 mμ, which diminished on the addition of cyanide (dotted line), indicates methemoglobin, while the diffuse absorption above 610 mμ even after cyanide indicates "sulfhemoglobin" and related compounds. Note the use of a logarithmic scale on the ordinate.

was carried out with the IRC resin column described above. Filter paper electrophoresis was performed with the glass plate apparatus (32) and with 0.05 M barbital buffer, pH 8.6. Hemoglobin specimens were treated with one heme-equivalent of sodium cyanide prior to application.

Three-times crystallized heme was provided by Dr. Rudi Schmid. Methemalbumin was prepared as described by Rosenfeld and Surgenor (33). Globin was produced from crystalline hemoglobin by the method of Rossi-Fanelli, Antonini and Caputo (34).

RESULTS

The effects of phenylhydrazine and acetylphenylhydrazine on hemoglobin

1. Spectrophotometric changes. The color changes produced in hemoglobin (either in solution or within red cells) by phenylhydrazine and related compounds varied with the dose and duration of the exposure (as shown below). The spectroscopic changes in hemoglobin solution exposed to 4 equivalents [4] of phenylhydrazine are shown in Figure 1. Phenylhydrazine caused a diminution of the double peaks of absorption, at 540 and 580 mμ, which are characteristic of oxyhemoglobin, and in larger doses (not shown here) caused their replacement with a single peak at 540 mμ. The increased absorption at 500 mμ and the presence of a peak at 635, which disappears after

[4] The calculations of mole equivalent are based on a molecular weight of 68,000 for hemoglobin, of 145 for phenylhydrazine hydrochloride, and of 146 for acetylphenylhydrazine.

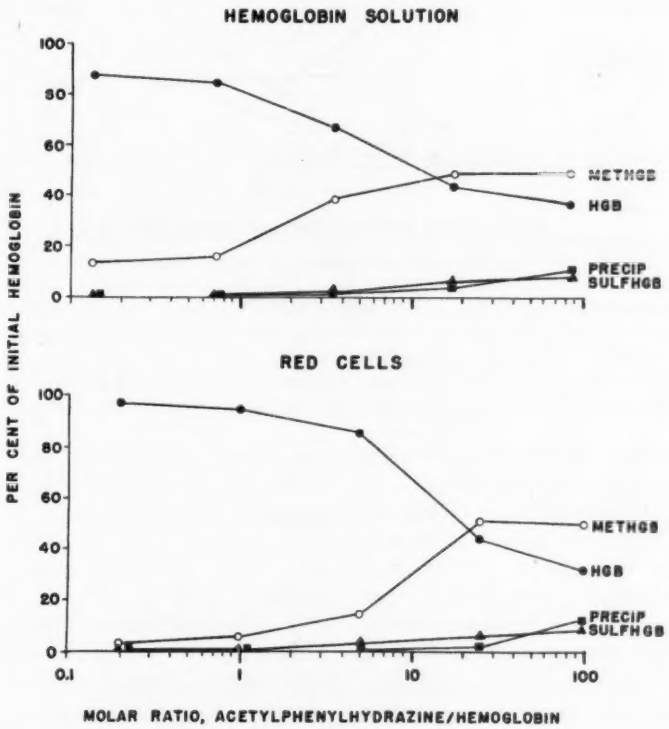


FIG. 2. EFFECTS OF VARIOUS QUANTITIES OF ACETYLPHENYLHYDRAZINE ON HEMOGLOBIN IN SOLUTION (ABOVE) AND WITHIN WASHED RED CELLS (BELOW). Apart from the fact that the solutions of crystalline hemoglobin contained 12 per cent methemoglobin to begin with, the levels of methemoglobin (empty circles), sulfhemoglobin (black triangles), and of hemoglobin (ferrohemoglobin) (black circles) were similar in the two preparations. In the red cell preparation the water-soluble precipitate value (black squares) was corrected for the weight of the stroma of untreated cells.

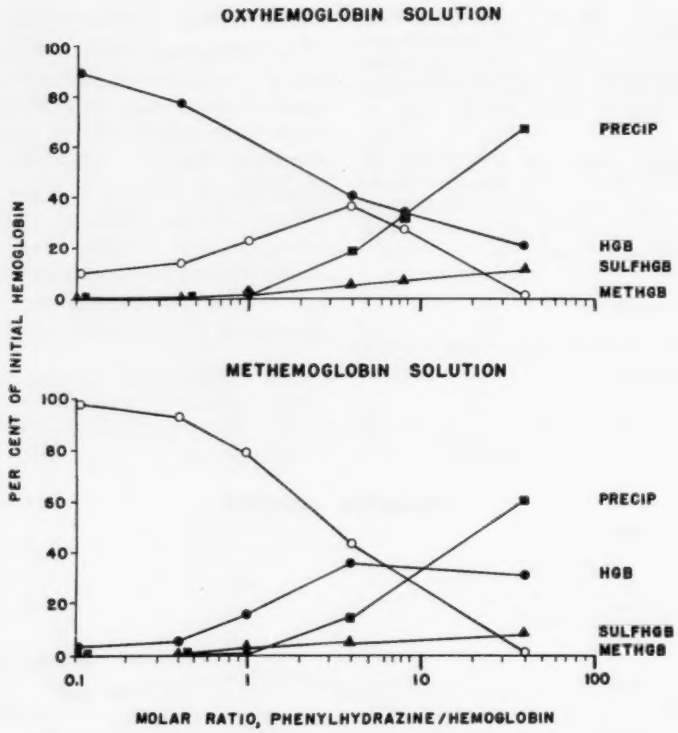


FIG. 3. EFFECTS OF VARIOUS QUANTITIES OF PHENYLHYDRAZINE ON SOLUTIONS OF OXYHEMOGLOBIN (ABOVE) AND OF METHEMOGLOBIN (BELOW). Maximum methemoglobin levels were attained with 4 volumes of phenylhydrazine per mole of oxyhemoglobin (i.e., the drug was equimolar with heme), at which dose the same oxyhemoglobin/methemoglobin equilibrium was attained whether the starting material was oxyhemoglobin or methemoglobin. At higher doses of drug methemoglobin was either reduced or precipitated.

cyanide, are characteristic of methemoglobin. The diffuse increase in absorbance above 610 mμ, declining gradually with increasing wave lengths and not removed by cyanide, is a feature of those irreversibly denatured hemoglobins, or hemochromagens, referred to earlier. Since in the Evelyn-Malloy procedure, employed in the kinetic studies below, the persistent absorption of hemoglobin derivatives at 620 mμ after cyanide is used to measure "sulfhemoglobin," and since there is no general agreement on the nomenclature of substances with this characteristic, the term sulfhemoglobin will be employed arbitrarily hereafter to indicate such substances.

2. The effect of dose. The effects of various amounts of acetylphenylhydrazine on hemoglobin solutions and on washed red cell suspensions during 2 hours' incubation at 37° C are depicted in Figure 2. In both preparations methemoglobin appeared within this period of time when equimolar amounts of the drug were used, while sulfhemoglobin and a precipitate appeared in appreciable quantities only at higher drug levels. The amount of methemoglobin produced reached a maximum in both preparations at a drug concentration of about 20 equivalents. No further increase was produced at drug levels of about 100 equivalents, although the amount of sulfhemoglobin and the weight of precipitate were increased.

A comparable study of the effects of various amounts of phenylhydrazine on solutions of crystalline hemoglobin is presented in the upper portion of Figure 3. With this agent methemoglobin was produced in appreciable amounts by as little

as 0.4 equivalent and reached a maximum at 4 equivalents of phenylhydrazine. At still higher levels of the drug the methemoglobin was reduced again to hemoglobin (see below). The maximum methemoglobin level corresponds to the point at which phenylhydrazine and heme are at equivalence. As with acetylphenylhydrazine, higher concentrations of drug were required to produce sulfhemoglobin and to precipitate the hemoglobin than those needed to produce methemoglobin; indeed, precipitation and sulfhemoglobin formation were greatest at levels of drug which had completely reduced the methemoglobin. Note that in a 2 hour period of incubation phenylhydrazine is considerably more potent than acetylphenylhydrazine in degrading hemoglobin.

3. Time relationship. The relative amounts of methemoglobin, sulfhemoglobin, and precipitated hemoglobin at various time intervals after the addition of 20 equivalents of phenylhydrazine or of

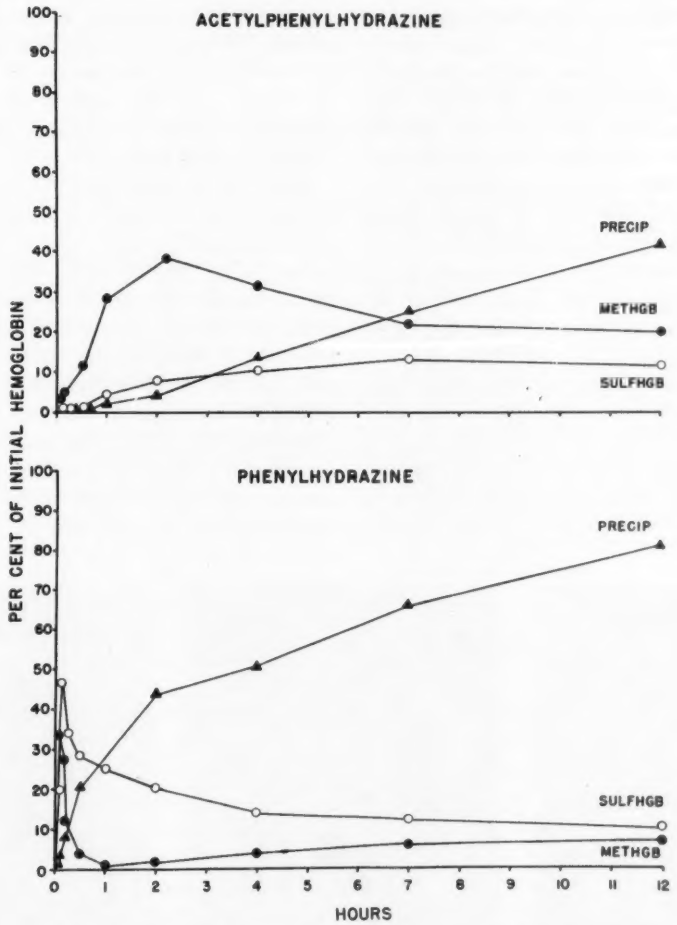


FIG. 4. SEQUENTIAL CHANGES IN HEMOGLOBIN EXPOSED TO ACETYLPHENYLHYDRAZINE (ABOVE) AND TO PHENYLHYDRAZINE (BELOW). Unlike phenylhydrazine, which causes immediate injury to solutions of hemoglobin, acetylphenylhydrazine becomes "active" only after a delay period. Note the early, transient appearance of methemoglobin followed by the later, transient appearance of sulfhemoglobin, and finally the conversion of pigment into a precipitate.

acetylphenylhydrazine per equivalent of hemoglobin are presented in Figure 4. It is apparent that phenylhydrazine acts rapidly in terms of pigment changes with an accompanying, but continuing, precepitation of hemoglobin. The concentrations of methemoglobin and sulfhemoglobin at this dose of phenylhydrazine were maximal within 1 minute of admixture; thereafter methemoglobin levels fell off rapidly and sulfhemoglobin levels declined gradually in reciprocal relation to the accumulating amount of precipitate. Acetylphenylhydrazine in the same concentration produced a gradual rise in methemoglobin levels, which reached a maximum in 2 hours and then declined, disappearaing completely within 20 hours (not shown). Sulfhemoglobin first appeared after 30 minutes' incubation, reached a maximum several hours later, and then declined gradually. Precipitated hemoglobin accumulated slightly more slowly at first, but steadily increased thereafter; by 20 hours (not shown) the total amount of hemoglobin precipitated reached 75 per cent of the hemoglobin initially present in solution, almost as much as was precipitated by phenylhydrazine.

4. The precipitate. That the precipitated material in the experiments cited above was precipitated hemoglobin, and not precipitated drug, is evident from purely gravimetric considerations. The precipitate was dark brown and granular in gross appearance. The granules tended to form uniform, small clumps and hair-like strands on standing. Although insoluble in water or 0.1 N NaCl the precipitate could be dissolved slowly in 0.1 N NaOH or 0.1 N HCl and readily dissolved in 8 M urea. It was solubilized within 2 to 3 hours by digestion at 37° C in 0.03 per cent crystalline trypsin in phosphate buffer, pH 7.8. Solutions of precipitate in 0.1 N NaOH showed a Soret-type absorption band at 405 mμ. This band was similar in position but only 54 per cent as intense as that of an equivalent amount by weight of oxyhemoglobin examined in the same solvent. A pyridine extract of the washed precipitate showed an intense absorption band at 558 mμ when reduced with sodium metabisulfite (forming a reduced pyridine hemochromagen) indicating the presence of heme.

Simultaneous studies of the effects of phenylhydrazine or acetylphenylhydrazine on red cells as compared to hemoglobin solutions revealed that Heinz bodies formed in the cells coincident with the appearance of a visible precipitate in the solution. Microscopic examination revealed a striking similarity in the appearance of the granules in red cells and the granules precipitated in hemoglobin solutions, as regards size, shape, aggregating characteristics, and staining qualities. In Figure 5 granules of hemoglobin precipitated by phenylhydrazine are compared with Heinz bodies produced in red cells at the same time by phenylhydrazine.

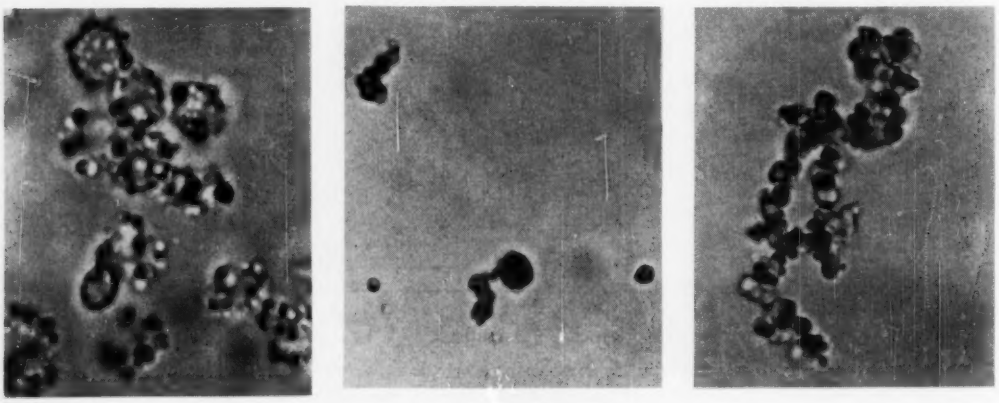


FIG. 5. COMPARISON OF PRECIPITATED HEMOGLOBIN AND OF HEINZ BODIES PRODUCED BY PHENYLHYDRAZINE. The photographs were taken of crystal violet-stained suspensions of red cells containing Heinz bodies (left) and of precipitated hemoglobin (middle and right) 2 hours after the addition of phenylhydrazine. The aggregate of unstained, precipitated hemoglobin granules at the right shows the characteristic tendency of these spheroidal bodies to align in irregular, branching chains. (Magnification × 2,000.)

The granules of precipitate varied in size from barely visible specks early in their formation to spherules 2 to 3 μ in diameter later on. These spherical particles tended to align themselves at first in short chains, then in irregular, branching chains, and finally in large aggregates. Some particularly long chains had the gross appearance of hair. The granules were identical in appearance to those Heinz bodies which had been extruded into the suspending medium from red cells. The conclusion seems warranted that Heinz bodies are themselves simply particles of precipitated, denatured hemoglobin and that their apparent structure simply reflects the physical configuration peculiar to precipitates of this protein.

The question as to whether this type of hemoglobin precipitate is peculiar to the effect of phenylhydrazine-like drugs was examined by precipitating hemoglobin with acid or with heat. Precipitation by exposure to 0.2 N HCl for periods of time, ranging between 30 seconds and 1 hour, before neutralization with 0.2 N NaOH, caused a precipitate of fine granules that resembled those appearing shortly after the addition of phenylhydrazine but that never achieved the size which the latter eventually attained. Precipitation of hemoglobin at 75° C caused granules to appear within 30 seconds that closely resembled those formed by phenylhydrazine, differing only in that there was little discoloration. Although heating at 75° C for 5 minutes produced a heavy, granular precipitate which stained blue with crystal violet, relatively little methemoglobin was produced and there was no sulfhemoglobin.

Human serum albumin and ovalbumin were not visibly affected by phenylhydrazine or acetylphenylhydrazine. Methemalbumin showed no tendency to precipitate in the presence of these drugs. Purified native globin spontaneously precipitated during incubation at 37° C under oxygen. The rate of this precipitation was not apparently influenced by phenylhydrazine. Except for its light color, the precipitate of globin was identical in appearance to that of drug-treated hemoglobin.

5. Electrophoretic and chromatographic changes. Both phenylhydrazine and acetylphenylhydrazine produced changes in the mobility of hemoglobin by paper electrophoresis at pH 8.6 (Figure 6). Both with red cells and with hemoglobin solutions, these drugs caused the appearance of a color peak

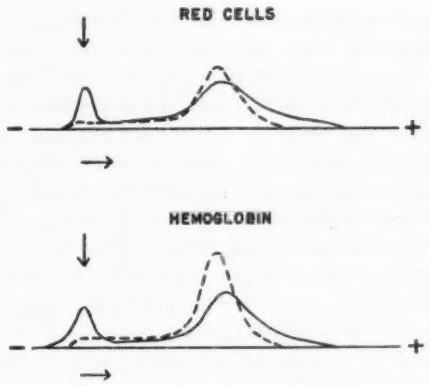


FIG. 6. CHANGES IN HEMOGLOBIN MOBILITY ON ELECTROPHORESIS AT pH 8.6 BEFORE (INTERRUPTED LINES) AND AFTER (CONTINUOUS LINES) THE ADDITION OF PHENYLHYDRAZINE. With hemoglobin either in red cells (above) or in solution (below), phenylhydrazine produced a greenish-brown peak which remained at the origin (beneath the vertical arrows) and a fast-moving component. The main peak was either unchanged in position or only slightly faster than normal.

which remained at the origin. This immobile peak was present even in clear, ultracentrifuged specimens, and was greenish-brown in color. The majority of the drug-treated hemoglobin was brownish-red (after cyanide) and showed approximately the mobility of normal hemoglobin; however, the color peak on electrophoretic strips tended to be slightly faster than normal (most evident in the lower portion of Figure 6) and a conspicuous fast-moving minor component was present. These changes appeared at concentrations of drug that produced methemoglobin with little or no sulfhemoglobin and no precipitate, and became more striking with bigger doses. Only with virtually complete precipitation of hemoglobin by phenylhydrazine did the mobile hemoglobin peak of the remaining soluble hemoglobin convert entirely into a fast-moving component; under these extreme conditions this peak was greenish-brown and broadly smeared.

After exposure to either phenylhydrazine or acetylphenylhydrazine there were changes in the behavior of hemoglobin on ion-exchange (IRC-50) resin columns. Normal hemoglobin on such a column can be separated into several fractions (30), including A_1, a front-running component which under standard conditions comprises about

10 per cent of the total and may include the fetal hemoglobins, and A_{II}, a slower-moving component comprising almost 90 per cent of the total. Although these fractions are not completely analogous to those found on electrophoresis, a portion of the front-running chromatographic fraction has an electrophoretic mobility corresponding to the "fast" hemoglobin of Kunkel and Wallenius (35). Hemoglobin treated with either of these two drugs showed an increase in the percentage of the front-running (A_I) fraction with a corresponding decrease in the main (A_{II}) component. This increased A_I fraction did not differ in its spectroscopic properties from the A_{II}. A_I and A_{II} fractions previously prepared by chromatographic separations were equally susceptible to degradation by phenylhydrazine. On exposure to phenylhydrazine Fraction A_{II} was almost entirely converted into A_I.

Factors influencing the action of phenylhydrazine-like drugs on hemoglobin

1. Temperature. At 0° C acetylphenylhydrazine produced no measurable change in the hemoglobin of either red cells or of hemoglobin solutions in the 2 hour period of observation.

2. Gas phase. Under nitrogen, using hemoglobin solutions largely depleted of oxygen by repeated flushing with nitrogen in a tonometer, phenylhydrazine and acetylphenylhydrazine caused partial reduction of the initial concentration of methemoglobin, some sulfhemoglobin appeared, but there was no precipitation. Under these conditions red cells did not develop Heinz bodies.

Under an atmosphere of pure carbon monoxide, phenylhydrazine reduced all of the original methemoglobin of hemoglobin solutions, traces of sulfhemoglobin appeared, but there was no precipitation and in red cells there were no Heinz bodies. In an atmosphere of 10 per cent carbon monoxide and 90 per cent oxygen, about 70 per cent as much precipitate formed as under 100 per cent oxygen.

3. Heme complexes. The experiment immediately above indicated that carbon monoxyhemoglobin was less susceptible than oxyhemoglobin to denaturative precipitation by phenylhydrazine even when oxygen was present in the gas phase. However, it is difficult to dissociate effects on hemoglobin due to the displacement of oxygen from those due to the stabilizing effect of carbon monoxide.

In the presence of a large excess of sodium cyanide (up to 100 equivalents relative to hemoglobin) the precipitative effect of acetylphenylhydrazine or of phenylhydrazine on hemoglobin in solution or in cells was only slightly diminished, and there was some increase in sulfhemoglobin as compared to the results when cyanide was omitted.

Solutions of oxyhemoglobin were compared with methemoglobin prepared by pretreating some of the hemoglobin solution with 1.2 heme-equivalents of potassium ferricyanide at pH 6.8, followed by dialysis. As shown in the upper part of Figure 3, almost 40 per cent of the hemoglobin of oxyhemoglobin solutions was oxidized to methemoglobin by 4 equivalents (1 heme-equivalent) of phenylhydrazine; at higher levels of this drug the methemoglobin concentration diminished along with the level of total soluble hemoglobin. When all of the initial hemoglobin was in the form of methemoglobin, as indicated in the lower part of Figure 3, the level of methemoglobin fell, rather than rose, on exposure to phenylhydrazine, so that once again at a dose of 4 equivalents of phenylhydrazine, approximately 40 per cent of the hemoglobin was methemoglobin. Higher concentrations of phenylhydrazine caused further reduction of the methemoglobin and total hemoglobin levels. Note that regardless of the oxidation state of the heme iron to begin with, 1 heme-equivalent of phenylhydrazine produced approximately a 50:50 equilibrium between ferroheme and ferriheme (hemoglobin and methemoglobin). There was no difference between these solutions in the total quantity of sulfhemoglobin or precipitate produced. When methemoglobin prepared with ferricyanide as described above was treated with 1.2 heme-equivalents of sodium cyanide prior to the addition of phenylhydrazine, none of the resultant methemoglobin cyanide was reduced by phenylhydrazine and no precipitate or sulfhemoglobin was formed, even under an atmosphere of 95 per cent oxygen.

4. Effects of preincubation of acetylphenylhydrazine. Although preincubation of acetylphenylhydrazine with oxygen and traces of metal ions caused a marked increase in its ability to oxidize glutathione (36), no such potentiation of its ability to precipitate hemoglobin could be demonstrated. Initially, slightly more methemoglobin was formed from hemoglobin treated with oxygen-preincubated

acetylphenylhydrazine than by the nitrogen-preincubated drug. When a 0.5 per cent solution of hemoglobin reacted with a 250 M excess of acetylphenylhydrazine preincubated under oxygen for 2 hours, 2 per cent methemoglobin formed in 30 seconds, whereas, when the acetylphenylhydrazine was preincubated under nitrogen, no methemoglobin formed within the first 50 seconds.

5. Comparative effect of several redox compounds. As noted above, phenylhydrazine caused a considerably more rapid destruction of hemoglobin than did acetylphenylhydrazine. Hydroxylamine, like phenylhydrazine, produced a rapid conversion of hemoglobin successively to methemoglobin and then to sulfhemoglobin and precipitated hemoglobin; *p*-aminophenol closely resembled phenylhydrazine in its effects on hemoglobin; *o*-aminophenol acted similarly in terms of methemoglobin formation but caused less sulfhemoglobin and precipitate. Aside from a slight in-

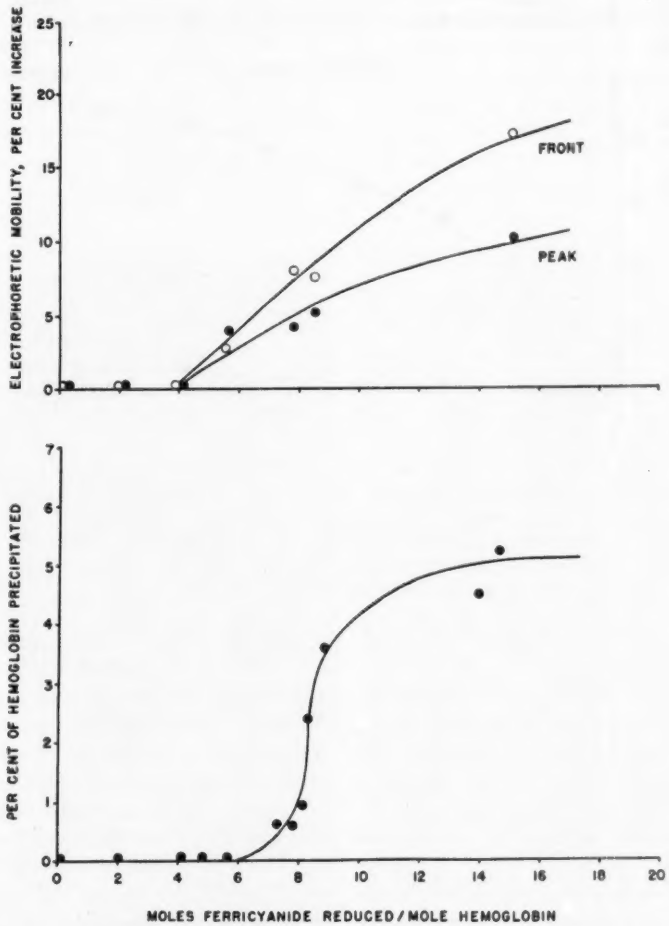


FIG. 7. CHANGES IN HEMOGLOBIN MOBILITY ON ELECTROPHORESIS AT pH 8.6 AND PRECIPITATION OF HEMOGLOBIN THROUGH ITS OXIDATION BY POTASSIUM FERRICYANIDE. As shown in the upper portion, there was an increase in the mobility of the fast-moving component ("front") and of the main component ("peak") of hemoglobin when more than four oxidation steps had occurred. Precipitation occurred (lower portion) when more than 6 equivalents of ferricyanide had been reduced by hemoglobin to ferrocyanide.

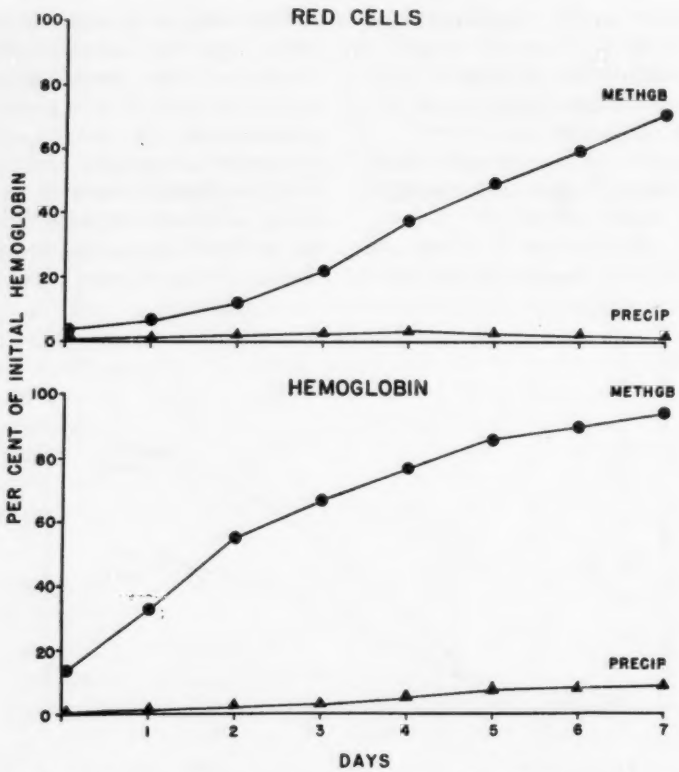


FIG. 8. EFFECT OF PROLONGED STERILE INCUBATION AT 37° C ON RED CELL SUSPENSIONS AND ON SOLUTIONS OF HEMOGLOBIN. In red cells (above) hemoglobin was converted to methemoglobin slowly during the first 2 days and then more rapidly and steadily thereafter. In solutions of hemoglobin (below) methemoglobin appeared at a rate of almost 1 per cent per hour for the first 2 days and then accumulated more slowly thereafter.

crease in methemoglobin, hemoglobin was unaffected by *m*-aminophenol.

6. Effect of "simple" oxidants. Potassium ferrocyanide, unlike some of the compounds above, is slow to auto-oxidize. Therefore, potassium ferricyanide is often employed stoichiometrically as a "simple" oxidant relative to heme. As expected, 4 moles of ferricyanide per mole of hemoglobin (1 heme-equivalent) converted all of the oxyhemoglobin to methemoglobin. At pH 6.8, where hemoglobin sulfhydryl groups are comparatively unreactive (37), 10 equivalents of ferricyanide added to hemoglobin produced no precipitate or sulfhemoglobin. At pH 9.1 in borate buffer 10 equivalents of ferricyanide produced in 3 hours at 37° C a precipitate equal to that caused by 4 equivalents of phenylhydrazine under the same conditions. While producing more methemoglobin, ferricyanide caused less sulfhemoglobin to appear than did phenylhydrazine. As did phenylhydrazine, ferricyanide caused an increase in the fast-moving component of hemoglobin on electrophoresis and on column chromatography.

In order to determine the number of oxidation steps necessary for the described changes in hemoglobin to occur, studies were conducted in which various amounts of ferricyanide were added to solutions of hemoglobin at pH 9.1, and the amount of methemoglobin and hemoglobin precipitate formed and the changes in the electrophoretic behavior of hemoglobin were compared with the number of equivalents of ferrocyanide elaborated. Ferrocyanide was measured by means of the Prussian blue reaction (37). As indicated

in Figure 7, no change occurred in the electrophoretic mobility of hemoglobin when less than 4 equivalents of ferrocyanide had formed; the first 4 equivalents of ferricyanide added had quantitatively oxidized the hemoglobin to methemoglobin as expected (*37*). With the reduction of slightly more than 4 equivalents of ferricyanide to ferrocyanide by hemoglobin, an increase in electrophoretic mobility became evident. When hemoglobin reduced more than 6 equivalents of ferricyanide, a gradual precipitation of hemoglobin ensued. Precipitation was inhibited by carbon monoxide. Under oxygen, precipitation of hemoglobin by ferricyanide was slower than by phenylhydrazine. These studies indicate that with the removal of two or more electrons from globin, changes occur in the hemoglobin molecule which permit its denaturative precipitation by oxygen.

The effect of prolonged incubation on red cells and hemoglobin

Under aseptic conditions washed red cells in isotonic phosphate buffer and solutions of crystalline hemoglobin were incubated for up to 7 days at 37° C. Part of the sequence of events is portrayed in Figure 8. Since with time the red cells underwent increasing degrees of "autohemolysis," the methemoglobin levels were determined on the washed cell suspension to exclude the influence of free hemoglobin. In the upper portion of the figure it is apparent that methemoglobin accumulates slowly during the first 24 to 48 hours and then increases arithmetically with time at the rate of approximately 10 to 12 per cent per day. Sulfhemoglobin (not shown) appeared after the fifth day, but never exceeded 3 or 4 per cent of the total. The "precipitate," the dry weight of the

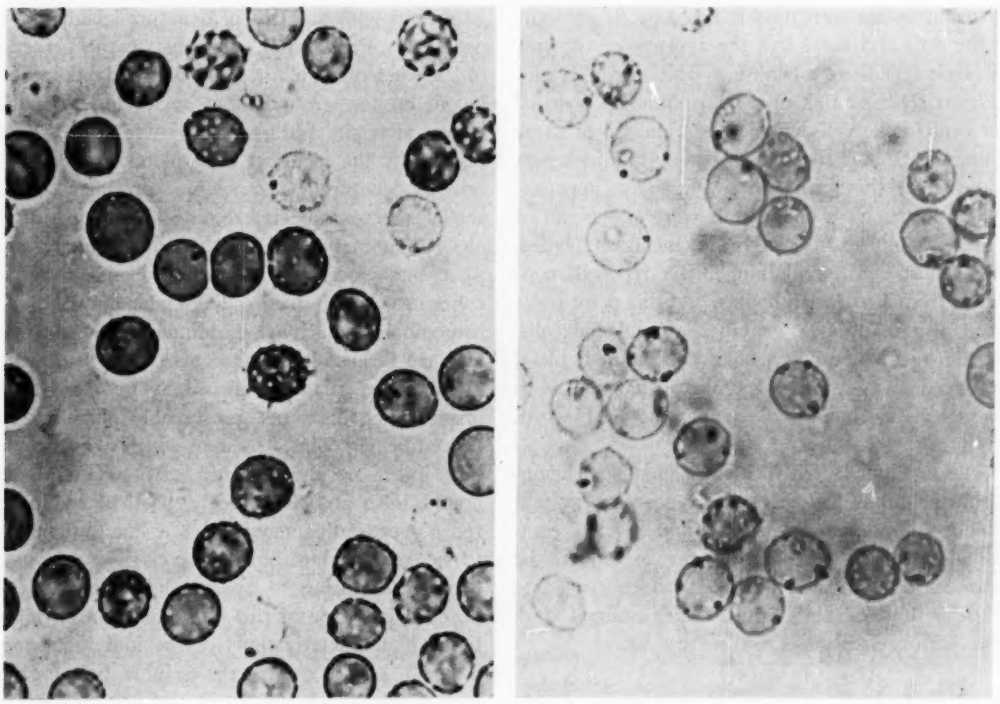


FIG. 9. APPEARANCE OF HEINZ BODIES IN NORMAL RED CELLS AFTER STERILE INCUBATION AT 37° C FOR 4 DAYS (LEFT) AND FOR 7 DAYS (RIGHT). At 4 days most of the cells are slightly irregular and crenated, showing refractile spicules and containing, in about one-quarter of the cells, one to three dark-staining inclusions resembling Heinz bodies. Virtually all ghosts, as shown in this picture, contained several such inclusions. After 7 days most of the cells (and all of those in the picture to the right) were ghosts, most of which contained several dark-staining inclusions resembling Heinz bodies. (Magnification × 2,000.)

sediment of the water hemolysate spun at 105,000 G for 1 hour, was corrected for the weight of the sedimented cell membranes on "Day 0". This precipitate tended to increase slowly during the first several days, and then to decline somewhat as cell membranes became more difficult to recover by centrifugation. Prolonged incubation of hemoglobin alone (lower portion of Figure 8) caused methemoglobin to form at the rate of about 18 per cent per day for the first 2 to 3 days, with a gradual decline in increment thereafter. Here again sulfhemoglobin (not shown) appeared after the third day in only trace amounts, never exceeding 3 per cent of the total. A slight precipitate was visible within 48 hours and this increased in amount steadily in the ensuing 6 days.

Microscopically, the red cells underwent increasing crenation during the first 2 days and thereafter ghosts became evident in small numbers. By the third or fourth day, from 1 to 3 inclusion bodies were visible in 20 or 30 per cent of the cells and these had the appearance of typical Heinz bodies on staining with crystal violet (Figure 9). At this time approximately 5 or 10 per cent of the cells were ghosts and all of these contained from 2 to 10 Heinz body-like inclusion bodies. By the sixth and seventh days approximately 70 per cent of the cells were ghosts, all of which contained from 2 to 15 inclusion bodies, while most of the remaining intact red cells contained from 1 to 4 such bodies. At this point there were also many free granules around the cells. Sterile incubation of whole defibrinated blood produced very similar pigment changes, with characteristic Heinz bodies appearing by the fifth day.

The precipitate that formed in incubated hemoglobin solutions had the same appearance as that produced by phenylhydrazine and related compounds; there were aggregates of spheroidal granules up to 1 μ in diameter, which tended to align with one another and which stained dark blue with crystal violet and methyl violet.

DISCUSSION

These studies indicate that phenylhydrazine and related redox compounds which can permeate the red cell membrane cause the same sequence of denaturative changes in hemoglobin, whether the hemoglobin is in pure solution or within red cells. This sequence involves *1*) spectrophotometric changes, *2*) electrophoretic and chromatographic changes, and *3*) precipitation of hemoglobin into coccoid bodies.

Spectroscopic changes. The earliest detectable change in hemoglobin on exposure to phenylhydrazine-like compounds *in vitro* is the appearance of a compound with the spectroscopic characteristics of methemoglobin. Since all of a large group of drugs tested by Emerson, Castle and Ham (17, 38) for hemolytic activity *in vitro* produced methemoglobin, it is probable that true methemoglobinemia occurs invariably in the early course of Heinz body anemias. Failure to observe methemoglobin in all instances (4) is probably attributable to its transience, due to the following: *1*) it is enzymatically reduced within the living cell (39); *2*) it reaches a redox equilibrium with compounds such as phenylhydrazine; *3*) it (in equilibrium with hemoglobin) bears a precursor relationship with a series of denatured hemoglobin products. Presumably, doubt as to the presence of methemoglobin in phenylhydrazine-treated blood, on the grounds that such blood remains brown after the addition of cyanide (40), is explained by the fact that such blood also contains brown denatured hemoglobin products which cause spectroscopic interference by their diffuse absorption in the red, and which gradually supplant methemoglobin at a rate dependent on the concentration of drug. These denatured brown hemoglobins, which are pyridine hemochromagens, and are termed here for convenience "sulfhemoglobin," are in turn replaced by poorly soluble, brown-green pigments, presumably including the "green hemoglobins," some of which may not contain intact porphyrin rings (3). These compounds also absorb diffusely, particularly in the red. Because this may in part be due to turbidity, particular care must be taken to clarify solutions of hemoglobin prior to spectrophotometry. It is beyond the scope of this work to attempt definitions or characterizations of the individual compounds. As a group, the soluble brown-green compounds which absorb in the red showed the following behavior: *1*) they followed methemoglobin in order of appearance; *2*) the color change was not reversed by cyanide, thiollic reduction, or dialysis; *3*) their appearance coincided with the appearance of insoluble hemoglobin products and with

soluble brown-green pigments which were immobile on electrophoresis at pH 8.6 and on ion exchange columns at pH 7.2. Many of our observations concerning the nature of these hemoglobin derivatives merely affirm previous studies by others, including Warburg, Kubowitz and Christian (40), Kiese (7), Heubner (41), and Beaven and White (8). Similarly, Mills (42) found that acetylphenylhydrazine added to rat hemoglobin led to a mixture of breakdown products of hemoglobin rather than to a single pigment such as choleglobin.

Changes in electrophoretic and chromatographic properties. Shortly before the first appearance of sulfhemoglobin-type pigments after treatment of red cells or hemoglobin solutions with phenylhydrazine or related compounds, there appeared on electrophoresis at pH 8.6 an increase in the fast-moving minor component of hemoglobin, which component did not differ in its spectral absorption pattern from the major hemoglobin component. Only with very large amounts of drug was the majority of hemoglobin converted to fast-moving hemoglobin on electrophoresis. Indeed, this occurred only when most of the hemoglobin had been precipitated. As further published work will show, this increase in electrophoretic mobility differs in certain respects from that produced by simple blocking of hemoglobin thiol groups (36), but resembles that produced by sodium chromate (43) and is similar to the fast component found in senescent red cells (35, 44).

The front-running fraction of hemoglobin on IRC resin column chromatography, previously suspected to contain oxidized sulfhydryl groups (30), includes the electrophoretically fast component of normal hemoglobin; this fraction is increased by phenylhydrazine and related compounds. Indeed, it was found that a purified preparation of the main component of hemoglobin A could be entirely converted into chromatographically fast hemoglobin by such compounds. It is of interest that on standing at room temperature pure preparations of the main component tend to convert spontaneously in part to the fast component.

Taken together, the facts above suggest that *in vivo* and *in vitro* hemoglobin tends with time to increase in charge density, that *in vitro* this process can be sharply accelerated by phenylhydrazine-like drugs, and that this change occurs just before and during manifestations of frank denaturation. An analogous increase in the electronegativity of albumin has been observed during the course of its denaturation (45).

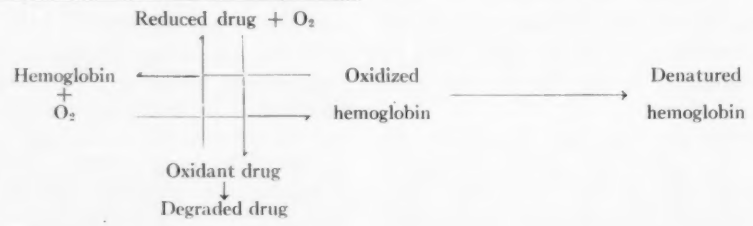
Shortly after the appearance of electrophoretically-fast hemoglobin and at the first appearance of pigments resembling sulfhemoglobin, there appeared a brown or brownish-green pigment that failed to move on electrophoretic strips or on the ion exchange column. This pigment (or pigments) was a pyridine hemochromagen and probably contributed to the absorption at 620 mμ. It was usually associated with the appearance of turbidity or of frank precipitation, and is presumed to be a partly soluble form of denatured hemoglobin.

The nature of Heinz bodies. In appearance and manner of evolution the precipitate of hemoglobin produced by phenylhydrazine-like drugs bore an unmistakable resemblance to Heinz bodies. Indeed, it is reasonable to conclude that Heinz bodies are simply granules of precipitated hemoglobin. Their relatively regular, micellar appearance seems to be determined by those general properties of hemoglobin which cause it to aggregate in a certain way when denatured, whether by these drugs, by heat, or by spontaneous aging. The point at which these spherical granules stop "growing," and their tendency to aggregate is fairly uniform, but is influenced by their rate of formation and by the presence of sulfhydryl compounds (36). It is probable that previous reports that Heinz bodies contain lipid or free iron (1, 2) reflect the tendency of these granules to adhere to neighboring structures as well as to each other. It is also perfectly possible that *in vivo* Heinz bodies may at times accumulate a mantle of other insoluble intracellular material. The suggestion by several workers (3, 12) that Heinz bodies contain or consist of denatured globin has been in essence verified, albeit in large measure the hemes remain attached, or are reattached. The precipitate had much the same color qualities as the soluble and partly-soluble brown or greenish-brown pigments described above and is presumably similar in nature to the greenish hemoglobin precipitate observed by Warburg and his associates (40) and by Beaven and White (8) when hemoglobin was treated with acetylphenylhydrazine. The latter

authors (8) concluded that the precipitate contained a mixture of protoheme and other iron-containing pigments bound to denatured globin.

The mechanism of drug action. The previous and present findings cited above are consistent with the proposition that phenylhydrazine and related drugs, in the presence of oxygen, cause a progressive, oxidative destruction of hemoglobin. The changes in hemoglobin may be characterized by the following stages: *1*) formation of methemoglobin, *2*) increase in electronegativity, *3*) formation of sulfhemoglobin-like hemochromes, and *4*) frank denaturative precipitation as Heinz bodies. These changes can also be brought about with "simple" oxidants, such as ferricyanide. That phenylhydrazine-like drugs cause an oxidative breakdown of hemoglobin has been postulated or accepted previously by a number of investigators (3, 8, 11, 12, 15, 41, 46).

As a class, drugs which induce Heinz body anemias are either actually, or potentially, highly reactive redox compounds. Those which are active *in vitro* tend (although at various rates) to oxidize spontaneously under oxygen into compounds which immediately oxidize hemoglobin to methemoglobin and GSH to GSSG. In terms of the latter reaction, at least, the rate of formation of these oxidant derivatives is catalyzed by hemoglobin (36); and it has been shown that hemoglobin in turn catalyzes the degradation of phenylhydrazine and other arylhydrazines to benzene and nitrogen (8), probably through a series of intermediate oxidized compounds, including phenylhydroxylamine, nitrosobenzene (47), hydroxylamine, and phenol (8). Since, to precipitate hemoglobin, these drugs require oxygen and the presence of a reactive heme group, since they enter into a dynamic equilibrium with heme, and since they exceed "simple" (i.e., slowly reversible) oxidants such as ferricyanide in their capacity to precipitate hemoglobin, it appears likely that: *1*) these compounds are actual, or potential, intermediates between oxygen and hemoglobin, and *2*) in the presence of oxygen these compounds and hemoglobin each catalyze the destruction of the other. Such a hypothesis has been advanced by several investigators including Lemberg and Legge (3), Beaven and White (8) and Emerson, Castle and Ham (17, 38). The latter authors (38) pointed out that most hemolytic drugs were resonating aromatic compounds, similar to the reversible oxidation reduction systems studied by Michaelis (48). Substances of this sort could speed the electron transfer from ferrous heme groups and from the sulfhydryl groups of the hemoglobin to molecular oxygen by providing partially oxidized intermediates, including free radicals, stabilized by resonance. Thus, after their conversion to quinone-like substances by molecular oxygen, they are capable of accepting single electrons in two successive steps to form: *1*) quinhydrone analogues, and *2*) hydroquinone analogues, while two ferrous heme or sulfhydryl groups are being oxidized. Oxygen itself can be partially reduced to form hydrogen peroxide, a compound more active as an oxidant of heme or of sulfhydryl groups than molecular oxygen and capable of accepting single electrons. However, as compared to the oxidant derivatives of phenylhydrazine and related compounds, hydrogen peroxide is very unstable, especially in the presence of substances with catalase activity. Thus, whereas hydrogen peroxide per se can simulate phenylhydrazine in its destruction of hemoglobin, it does so only when in relatively large amounts in the presence of both oxyhemoglobin and cyanide, the latter presumably blocking the catalase activity of methemoglobin. It seems unlikely, despite previous speculations (41, 49), that the active derivative of these drugs is hydrogen peroxide itself. A simple schema as to the probable sequence of events is presented:



If the effect of these oxidant drugs is then to expedite transmission of the high oxidation potential of molecular oxygen it should be possible to demonstrate that the same changes occur, although more slowly, when hemoglobin is exposed to oxygen alone. Such was indeed the case, for on prolongd incubation under oxygen at 37° C, but not at 0° C, solutions of hemoglobin went through, in several days, a sequence similar to that occurring with phenylhydrazine in minutes: conversion to methemoglobin, appearance of "sulfhemoglobin" and a fast-moving component on electrophoresis, and finally precipitation as Heinz body-like granules. Red cells so incubated manifested the same sequence of pigment changes and developed Heinz bodies on the fourth or fifth day. These changes occurred under oxygen but not under carbon monoxide. The spontaneous appearance of inclusion bodies in blood stored for several days has been previously observed by others (50–53). Most of these granules appeared either in reticulocytes (50, 51) or represented siderocytes (53), in contrast to the present studies in which the granules occurred in adult red cells and did not stain for iron. Moeschlin (52), on the other hand, observed small, nonsiderotic inclusion bodies in adult red cells which had been incubated for 2 to 3 days at 37° C, but not at 0° C, and noted that these inclusions were similar to Heinz bodies in several respects. Because of their marginal placement he termed these bodies "Randkörperchen" and suggested that their presence may presage the death of the red cell.

Whether or not Heinz bodies form during normal red cell aging *in vivo,* as they do *in vitro,* is not presently known. Since frank precipitation of hemoglobin may be considered an advanced stage of injury, which may be preceded by more subtle but significant cellular alterations, it seems likely that the spleen, and possibly other elements of the reticuloendothelial system, may remove red cells so injured from the circulation and prevent their accumulation there, provided the number of cells is not too great. It is of interest in this connection that red cells containing Heinz bodies have been reported as a normal finding in splenectomized animals (54) and man (55, 56) and in patients with splenic agenesis (57). Therefore, it seems a reasonable possibility that normal aging *in vivo* is associated with the appearance of Heinz bodies.

Most of the observations reported here have been concerned with the spontaneous or accelerated oxidation of hemoglobin, because that protein is more accessible to observation than are structural components of the cell. The question as to how these observations bear on the fate of the surface and structure of the cell membrane, which determines the survival of the cell in circulation, can at present only be arrived at by analogy. Teleologically it would be desirable to dispense with a red cell containing denatured hemoglobin or hemoglobin with abnormal oxygen-dissociation properties. There is good evidence that the integrity of the sulfhydryl groups of hemoglobin is vital to the normal oxygen-dissociation characteristics of hemoglobin (58) and that these groups are involved in the degradation of hemoglobin by oxidants and by prolonged stasis (28, 30, 36). There is also evidence by Beutler and his associates (19, 27) that free intracellular thiols, chiefly glutathione, are oxidized or destroyed by oxidant drugs and are probably involved in the intracellular defense against these drugs. The work of Mills and Randall (11, 42, 59) indicates that GSH may protect hemoglobin from oxidation by serving as an oxidizable substrate in the presence of an enzyme, glutathione peroxidase. However, at least part of the protective effect of GSH is nonenzymatic and involves defense of the hemoglobin sulfhydryl groups (36). Finally, it has been shown that oxidation or blocking of red cell thiols is associated with increased membrane permeability (60) and with frank hemolysis *in vitro* (61–64). It may be, therefore, that thiol oxidation represents a central phenomenon which couples the destruction of hemoglobin with that of the red cell itself. Thus the red cells of "glutathione-unstable" subjects, and perhaps those of patients with spontaneous Heinz body (65, 66) or similar inclusion body (67, 68) anemias may undergo a form of spontaneous, premature senescence. Such a process may, or may not be sufficiently severe to cause anemia in the absence of drugs, and its morphologic recognition (e.g., the identification of inclusion bodies) might be possible only in the absence of the spleen. Recent evidence by Brewer, Tarlov, Kellermeyer and Alving (69) indicates

that the red cell lifespan in "glutathione-unstable" Negroes is significantly shorter than normal even in the absence of drug ingestion. The pattern of increased destruction is consistent with accelerated red cell senescence.

Red cell senescence has many features in common with senescence in another relatively inactive tissue, the lens of the eye. Like red cells, the lens contains a rich supply of GSH which falls during senility (70). As in red cells senescence is associated with a proteinaceous precipitate, the incipient cataract (70); as in red cells this event is presaged by a fall in GSH levels (71); and as in red cells this sequence of events may develop through interference with the pentose phosphate pathway, as by galactose (72, 73), or through exposure to an oxidant drug known to cause Heinz body anemias, naphthoquinone (74).

The observations here reported emphasize the fact that for its survival the red cell and its vital constituent, hemoglobin, are dependent upon certain special mechanisms for their common defense against the great oxidative potential of the very substance they are designed to transport—molecular oxygen. One of the seemingly essential features of this arangement is that there be a metabolic hiatus between oxygen and the cellular constituents, with an electron exchange sufficient only to provide the energy for certain necessary functions, including generation of TPNH for the reduction of glutathione. When this hiatus is bridged by the entrance of a redox intermediate into the red cell, when the cell's TPN-reducing apparatus has failed, or especially when both conditions coincide, the oxidative destruction of the cell is ordained.

SUMMARY

Phenylhydrazine and related redox compounds which cause Heinz body anemias *in vivo* were studied as to their effects *in vitro* on red cells and on solutions of crystalline human hemoglobin. With both substrates the following sequence of changes in hemoglobin were observed: *1*) methemoglobin was formed until a new, hemoglobin ⇌ methemoglobin equilibrium was achieved; *2*) a fast-moving component of hemoglobin on electrophoresis and resin column chromatography appeared; *3*) a group of soluble, poorly soluble, and insoluble brown to green "sulfhemoglobin-like," denatured pigments appeared; *4*) these denatured products of hemoglobin precipitated into coccoid bodies ranging up to 2 or 3 μ in diameter with properties identical to those of Heinz bodies. It is concluded that Heinz bodies represent granules of precipitated hemoglobin.

Drugs and chemicals active in this respect have the property of reacting with molecular oxygen, probably to form oxidant intermediates or free radicals capable of oxidizing hemoglobin and other intracellular components. These drugs thereby transmit the high oxidation potential of oxygen to cellular components in a cell containing uniquely high concentrations of oxygen and a substance, hemoglobin, which catalyzes the activation of the drug. In the course of several days red cells or hemoglobin incubated under oxygen alone underwent the same changes, including Heinz body formation, as those which occurred in minutes or hours in the presence of the oxidant drugs. Since some of these changes have been identified with aging *in vivo*, it appears that senescence, in the red cell at least, involves the irreversible oxidation of hemoglobin and of other cellular constituents. The Heinz body anemias appear to represent an acceleration of the normal processes of red cell aging.

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EFFECTS OF LONG-TERM STORAGE ON HUMAN SERUM ALBUMIN. I. CHROMATOGRAPHIC AND ULTRACENTRIFUGAL ASPECTS

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The problem of the stability of normal human serum albumin during storage under various conditions is of considerable practical as well as theoretical interest. The present report covers one phase of a long-term project undertaken in our laboratory to determine what changes occur in stored albumin, the conditions affecting these changes, and the most suitable methods for detecting protein alterations.

MATERIALS AND METHODS

Samples. The samples examined were vials of commercial normal serum albumin (human) from four lots prepared for administration to patients. The preparation involved ethanol fractionation of human plasma by method 6 of Cohn and co-workers (1) and subsequent heating, in the presence of stabilizers, at 60° C for 10 hours to kill the serum hepatitis virus (2). For two of the lots, starting material for fractionation was liquid citrated whole plasma; for one lot it was dried, ultraviolet-irradiated plasma; for one lot it was a mixture of both types of material.

Sterile albumin samples from each of three lots were stored for 5 years at 5° and 32° C, respectively. One lot was stored for 5 years at 5° C, whereupon the vials were divided into two groups for an additional 5 year storage period at 5° and 32° C, respectively. Most of the samples examined in this study were stored as 25 per cent protein solutions. A few samples of albumin stored in other states were also investigated.

Chromatography. Albumin solutions containing 500 mg of protein were diluted to 10 ml with "starting buffer" and dialyzed in the cold for 48 to 72 hours against four changes, approximately 750 ml each, of the same buffer. "Starting buffer" (pH 8.6) was 0.005 M in H_3PO_4 and 0.029 M in Tris(hydroxymethyl)-aminomethane (Tris). The final buffer was 0.50 M in both H_3PO_4 and Tris, and had a pH of 4.1. After dialysis the albumin samples were placed on a 2.2 × 35 cm column of diethylaminoethyl (DEAE) cellulose equilibrated with starting buffer. The DEAE cellulose, containing 0.78 mEq N per g, was prepared by the method of Peterson and Sober (3) from powdered cellulose,[1] 100 to 230 mesh.

[1] Solka-Floc, Brown Company, Berlin, N. H.

The chromatography was done at 6° C, essentially by the method of Sober and Peterson (4). A compound concave elution gradient was obtained with a nine-chambered gradient elution device ("Varigrad") of the type described by Peterson and Sober (5). Solutions placed in the chambers consisted of various amounts of final buffer diluted to 220 ml with starting buffer. The volumes per cent of final buffer in successive chambers were 0, 1.6, 2.0, 9.0, 9.0, 2.0, 20.0, 100, and 100. A flow rate of 72 ml per hour was used and 12-ml fractions were collected. The fractions were read at 280 mμ in a Beckman DU spectrophotometer.

Paper electrophoresis. Fractions from the column were placed in a negative pressure dialysis apparatus[2] which permitted simultaneous concentration of the protein and dialysis against isotonic saline. During this procedure the temperature was kept low by surrounding the apparatus with ice.

Paper electrophoresis was run on the concentrated samples in a Spinco Model R cell using barbital buffer, pH 8.6, ionic strength 0.075, on Whatman 3 MM filter paper at room temperature. Five ma per cell was applied for 18 hours, after which the strips were stained with bromphenol blue.

Ultracentrifugation. Fractions from the column, concentrated as described above, were dialyzed for 16 hours in the cold against at least 500 volumes of phosphate-buffered saline, pH 7.2, ionic strength 0.154. They were then centrifuged at 59,780 rpm for 50 to 100 minutes at 20° C in a Spinco Model E ultracentrifuge at a protein concentration of approximately 1 per cent. Relative amounts of the various components were determined by planimetry following completion of the curves in the schlieren pattern. Sedimentation coefficients were reported as s_{20} values.

RESULTS

While the elution profile of each sample differed to a greater or lesser extent from all others and the profiles probably represent a series of progressive changes rather than discrete types, they have been classed for convenience into four general types (Figure 1). The types exhibited by the

[2] Membranfiltergesellschaft, G.m.b.H., Göttingen, Germany. Distributed by Carl Schleicher and Schuell Co., Keene, N. H.

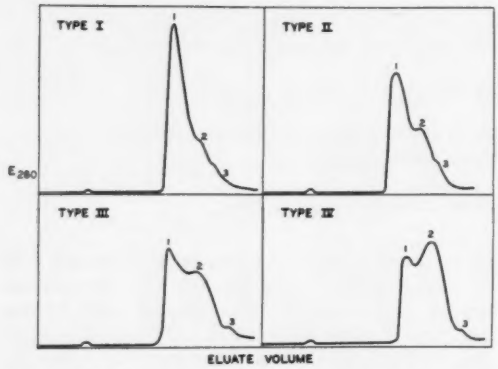

FIG. 1. TYPICAL CHROMATOGRAPHIC ELUTION PROFILES OF ALBUMIN SAMPLES.

various samples are summarized in Table I. All samples except those falling into type I showed two distinct peaks, usually with a small "foot" following them. These regions have been numbered 1, 2 and 3 (Figure 1). The small peak early in the patterns was shown to be due to acetyltryptophan which is added as a stabilizer and is not completely removed by dialysis, even after 72 hours. It was found in all the commercial samples, being absent only in the elution pattern of the fresh fraction V which was prepared in our laboratory by method 6 (1) and had not been stabilized or heated.

Storage of albumin, prepared from citrated whole plasma, for 5 years at 5° C resulted in only minimal alterations in chromatographic behavior (Table I). This was true whether it was stored as a 5 or a 25 per cent protein solution. On the other hand, after storage at 5° C of albumin from ultraviolet-irradiated dried plasma, or from a mixed source of irradiated dried and liquid plasma, or after storage for an additional 5 years of albumin from citrated whole plasma, a considerable quantity of material appeared in region 2. Storing at 32° C caused a fairly uniform change in the chromatographic pattern, although changes following storage as a 5 per cent solution were somewhat less drastic; storing at 32° C in the dry state seemed particularly deleterious (Figure 1 and Table I).

Paper electrophoresis of concentrated fractions revealed no difference between regions 1 and 2, both migrating like the albumin in freshly drawn plasma. Region 3, even when highly concentrated, appeared as a diffuse smear between the origin and the position of α_2-globulin. In samples showing type I elution profiles, any α-globulin present began to come off the column toward the end of region 2.

The protein in region 1 of all preparations had a sedimentation coefficient closely approximating the accepted value for human albumin (Table II). That this material was relatively homogeneous was evidenced by the slow spreading of the boundary during the course of ultracentrifugation.

Ultracentrifugation of all samples from region 2 revealed at least two boundaries (Table II). In materials with type IV chromatographic patterns, three boundaries were seen. In all of these, the slowest boundary had a sedimentation coefficient very close to that of region 1. The sedimentation coefficients of the next boundary were by no means constant among the various preparations, ranging from 5.90 to 7.00 S (Table II). In the two type IV patterns, the third ultracentrifugal peak had a still higher sedimentation coefficient.

TABLE I

Summary of chromatographic elution profiles

Lot	Source*	Per cent protein	Years stored	Type of elution pattern	
				5° C storage	32° C storage
Fresh fraction V, never heated	CW	Dry	0	I	
Fresh commercial albumin	CW	25	0	I	
203	CW	25	5	I	III
203	CW	5	5	I	II
204	CW + ID	25	5	II	III
205	ID	25	5	II	III
204	CW + ID	Dry	5	II	IV
201	CW	25	10	II	IV†

* CW = citrated whole plasma; ID = ultraviolet-irradiated, dried plasma.
† Stored 5 years at 5° C, then 5 more years at 32° C.

TABLE II

Ultracentrifugal analyses of chromatographic fractions

Lot	Source*	Years stored	Storage temp (° C)	Chromatog. pattern	s_{20} values in chromatographic regions†		
					Region 1	Region 2	Region 3
Fresh fraction V	CW	0		I	4.18	4.44, 7.13 (89%), (11%)	
			5	I	4.05	4.05, 5.90 (87%), (13%)	4.19, 7.54, 29.4 (83%), (13%), (4%)
203	CW	5	32	III	4.15	4.19, 6.68 (79%), (21%)	
			5	II	4.12	4.40, 6.73 (80%), (20%)	4.30, 6.44, 22.7 (67%), (6%), (29%)
204	CW + ID	5	32	III	4.15	4.09, 6.36 (80%), (20%)	4.55, 9.62, 33.4, (80) (59%), (11%), (31%), (tr.)
			32 Dry	IV	4.12	7.27 > 7.00 > 3.44	
201	CW	10	5	II	4.20	4.37, 6.64 (76%), (24%)	
			32‡	IV	4.15	4.19, 6.11, 8.34 (78%), (22%), (tr.)	

* CW = citrated whole plasma; ID = ultraviolet-irradiated, dried plasma.
† s_{20} values are in S; figures in parentheses are relative proportions of the appropriate components.
‡ Five years at 5° C followed by 5 years at 32° C.

Three region 3 samples were also ultracentrifuged. In all cases at least three boundaries were noted, one with a sedimentation coefficient greater than 22 S, and one with that of albumin. The relative proportion of fast component seemed to increase with increased change in the chromatographic pattern, types II and III having more than type I (Table II). A similar situation seemed to obtain in the case of the components from region 2 having sedimentation coefficients of 5.90 to 7.00 S. The increases in both cases were most marked between types I and II.

DISCUSSION

A number of workers (6–9) have utilized chromatography on modified cellulose to investigate macromolecular changes; two of these (7, 8) employed continuous gradient elution systems. Keller and Block (9) reported that when bovine mercaptalbumin was chromatographed on DEAE cellulose, the material with higher $s_{20,w}$ values was eluted at higher ionic strengths than were those components having $s_{20,w}$ values of albumin. In our studies, components with higher s_{20} values consistently came off the column in regions 2 and 3.

The present chromatographic work indicates that the extent of changes occurring in albumin during storage are dependent on both the nature of the material used for preparation, and the conditions of storage. The albumins which were chromatographically most similar to fresh fraction V were those prepared from liquid citrated whole plasma and stored at 5° C. The concentration during storage seemed to be of less significance, at least during the first five years.

The fact that when chromatographically detectable changes were seen, they always appeared in the same region of the pattern suggested that the changes were of a similar nature and progressed with the degree of protein alteration. However, a lack of uniformity in the nature of these changes is indicated by the wide range of sedimentation coefficients of the components with s_{20} greater than 5.9 S. The only consistent trend seems to be the appearance of more rapidly sedimenting material in chromatographic regions 2 and 3 as the elution profile type progresses.

Concomitant use of both chromatography and ultracentrifugation permitted measurement of components not observed by using either technique alone on whole commercial albumin. This was shown in parallel measurements on both 5° and 32° C samples of lot 204. Full studies of albumin storage using electrophoresis, viscosity, and other methods will be reported in the future. The indications are, however, that the chromatography-ultracentrifugation technique is at least as sensi-

tive for detection of protein change as other commonly used methods, and is probably more sensitive than most.

SUMMARY

Studies by means of chromatography on DEAE cellulose and subsequent ultracentrifugation were carried out on commercial human albumin preparations stored under various conditions for at least five years. Albumins prepared from citrated whole plasma and stored for five years at 5° C showed minimal difference in chromatographic behavior from fresh albumin samples. Those stored for ten years, stored at 32° C, or prepared from ultraviolet-irradiated dried plasma demonstrated more marked differences on chromatography, namely, increased amounts of material appearing later in the elution patterns. This latter material contained components with sedimentation coefficients greater than 5.9 S, the amounts of which seemed to increase with increasing change in the chromatographic profile. In this study coordinated use of both chromatography and ultracentrifugation provided an extremely sensitive method for detecting protein alteration.

ACKNOWLEDGMENT

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ESTIMATION OF PULMONARY ARTERIOVENOUS SHUNT-FLOW USING INTRAVENOUS INJECTIONS OF T-1824 DYE AND KR^{85}*

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While the concept of venous admixture is simple, the pathways responsible for this entity are complex (1–4). They include a multiplicity of channels which can allow venous blood to reach the peripheral circulation: among them are congenital anomalies of the heart and great vessels; bronchial, Thebesian and anterior cardiac veins; portal-mediastinal-pulmonary venous anastomoses; and pulmonary arteriovenous shunts. This last pathway is of particular interest because such shunts are thought to develop in several common diseases of man. But no method has been available for assessing the flow which they carry. Hence, the argument for their importance has been based largely on inferential evidence.

This paper has two purposes. The first is to describe a method which will detect, and under certain conditions estimate, abnormal amounts of pulmonary arteriovenous shunt-flow. The second is to present the results obtained when the method was applied in patients with either pulmonary emphysema, pulmonary infection, or alcoholic cirrhosis of the liver, three conditions in which these shunts are said to occur.

METHOD

The method entails: *a*) dissolving a small volume of the inert, gaseous isotope Kr^{85} in T-1824 dye, *b*) injecting a few cubic centimeters of this mixture into one of the great veins, and *c*) determining the ratio of dye to Kr^{85} concentrations in the systemic arteries during the initial passage of the indicators. Provided the patient is free of anomalies of the heart and great vessels, the ratio of concentrations depends on the partition of flow between alveolar capillaries and pulmonary arteriovenous shunts. The reasons for this dependence are depicted schematically in Figures 1, 2 and 3.

Figure 1 shows the concentration curves of dye and Kr^{85} in the blood leaving the alveolar capillaries. Because the dye remains in the blood stream, its curve has a characteristic contour which rises sharply, reaches a peak, and then declines. By way of contrast, more than 95 per cent of the Kr^{85} escapes into the alveoli (5), causing that remaining in solution to inscribe a curve which rises slowly to a maximum value occurring at about the point where manifest recirculation of dye appears.

Figure 2 compares the two curves when the indicators traverse pulmonary arteriovenous shunts. Since blood perfusing these channels is deprived of contact with alveolar gas, the Kr^{85} remains in the blood stream and the dye and Kr^{85} curves have similar forms.

Figure 3 depicts the curves obtained in the presence of both capillary and shunt pathways. In this situation the concentration of Kr^{85} in the common channel represents the sum of two components, namely, the sloping line from the capillaries and the peaked curve from the shunts.

Calculations

These considerations provide a background for estimating the pulmonary arteriovenous shunt-flow, and, as a beginning, it will be temporarily

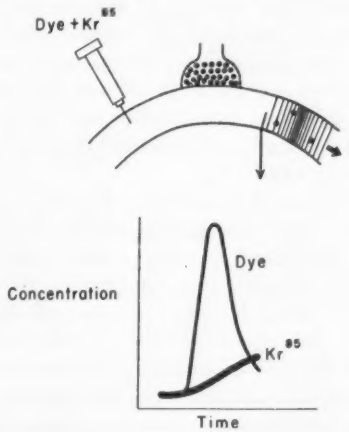

FIG. 1. SCHEMATIC DRAWING OF DYE AND Kr^{85} CURVES IN BLOOD LEAVING ALVEOLAR CAPILLARIES. Hatching represents dye, closed circles Kr^{85}.

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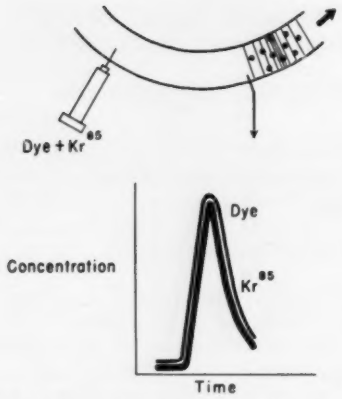

FIG. 2. SCHEMATIC DRAWING OF DYE AND Kr^{85} CURVES IN BLOOD LEAVING PULMONARY ARTERIOVENOUS SHUNTS. Hatching represents dye, closed circles Kr^{85}.

presumed that blood can be sampled at the point marked A in Figure 4. Thus, if blood is withdrawn at a constant rate during the first circulation of the indicators, the ratio of dye to Kr^{85} in the collected specimen is expressed by Equation 1.

$$\frac{Cc_D}{Cc_K} = \frac{\int_0^T Ca_D dt}{\int_0^T Ca_K dt} \qquad [1]$$

where Cc_D = concentration of dye in the specimen, Cc_K = concentration of Kr^{85} in the specimen, Ca_D = instantaneous concentration of dye in the vessel, Ca_K = instantaneous concentration of Kr^{85} in the vessel and T = time during which blood is sampled. The numerator of the right hand member represents the sum of the contributions of dye from the alveolar and shunt pathways; the denominator represents the sum of the contributions of Kr^{85}.

Assumptions of the method. The first assumption of the method is that the transit times of the alveolar and shunt pathways are equal. This assumption implies that the concentrations of dye in the blood leaving the two pathways are identical and equal to that in the confluent stream. By way of contrast, the concentration of Kr^{85} at the confluence can be described only by using the familiar mixing equation shown below.

$$Ca_K = \frac{\dot{Q}_s}{\dot{Q}_p} Cs_K + \left(1 - \frac{\dot{Q}_s}{\dot{Q}_p}\right) CA_K \qquad [2]$$

where Cs_K = instantaneous concentration of Kr^{85} in the blood from the shunts, CA_K = instantaneous concentration of Kr^{85} in the blood from the alveoli, $\dot{Q}_s$ = flow through shunts and $\dot{Q}_p$ = flow through the pulmonary artery. Substitution of Equation 2 in Equation 1 yields

$$\frac{Cc_D}{Cc_K} = \frac{\int_0^T Ca_D dt}{\int_0^T \left[\frac{\dot{Q}_s}{\dot{Q}_p} Cs_K + \left(1 - \frac{\dot{Q}_s}{\dot{Q}_p}\right) CA_K\right] dt}.$$

With $\dot{Q}_s/\dot{Q}_p$ constant during the time of the first circulation, this relation can be simplified to Equation 3.

$$\frac{Cc_D}{Cc_K} = \frac{\int_0^T Ca_D dt}{\frac{\dot{Q}_s}{\dot{Q}_p}\int_0^T Cs_K dt + \left(1 - \frac{\dot{Q}_s}{\dot{Q}_p}\right)\int_0^T CA_K dt} \qquad [3]$$

The second assumption of the method is that the ratio of concentrations of dye to Kr^{85} in the shunted blood is equal to the ratio of concentrations in the injectate. Hence

$$\frac{Cs_D}{Cs_K} = \frac{CI_D}{CI_K}$$

or

$$Cs_K = \frac{CI_K}{CI_D} \cdot Cs_D \qquad [4]$$

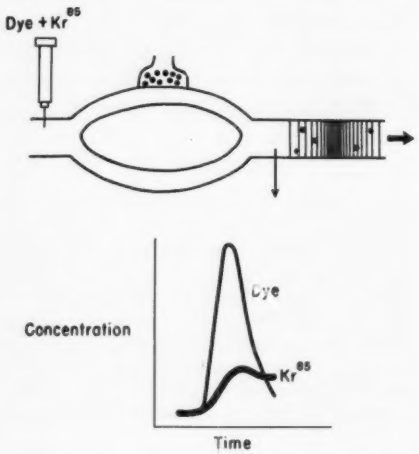

FIG. 3. SCHEMATIC DRAWING OF DYE AND Kr^{85} CURVES IN BLOOD AT CONFLUENCE OF STREAMS FROM ALVEOLAR CAPILLARIES AND PULMONARY ARTERIOVENOUS SHUNTS. Hatching represents dye, closed circles Kr^{85}.

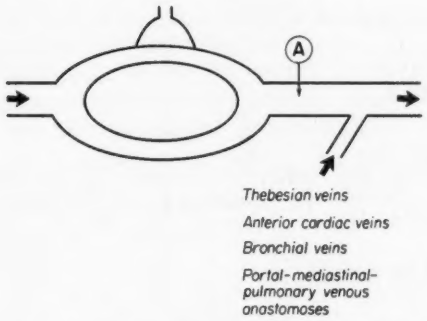

FIG. 4. SCHEMATIC DRAWING OF ALVEOLAR CAPILLARIES, PULMONARY ARTERIOVENOUS SHUNTS, AND CHANNELS CARRYING VENOUS ADMIXTURE ORIGINATING FROM SITES OTHER THAN THE PULMONARY ARTERY.

where C_{I_K} = concentration of Kr^{85} in the injectate and C_{I_D} = concentration of dye in the injectate. Substitution of Equation 4 in Equation 3 yields

$$\frac{C_{c_D}}{C_{c_K}} = \frac{\int_0^T C_{a_D}dt}{\frac{\dot{Q}_s}{\dot{Q}_p}\cdot\frac{C_{I_K}}{C_{I_D}}\int_0^T C_{s_D}dt + \left(1-\frac{\dot{Q}_s}{\dot{Q}_p}\right)\int_0^T C_{A_K}dt} \quad [5]$$

and, on the basis of the initial assumption that C_{a_D} and C_{s_D} are equal, C_{a_D} can be substituted for C_{s_D} in Equation 5.

$$\frac{C_{c_D}}{C_{c_K}} = \frac{\int_0^T C_{a_D}dt}{\frac{\dot{Q}_s}{\dot{Q}_p}\cdot\frac{C_{I_K}}{C_{I_D}}\int_0^T C_{a_D}dt + \left(1-\frac{\dot{Q}_s}{\dot{Q}_p}\right)\int_0^T C_{A_K}dt}. \quad [6]$$

Thus far the analysis has ignored the other sources of venous admixture, and it is now necessary to consider how the blood which they contribute influences the relation expressed in Equation 6. These sources, listed in Figure 4, convey venous blood into the pulmonary circulation or left heart chambers from vessels other than the pulmonary artery. Because this blood is free of dye and Kr^{85} during the initial passage of the indicators, it acts as a diluent and reduces the concentrations of dye and Kr^{85} equally. Hence, the ratio of concentrations of the indicators is insensitive to its presence, and the relation expressed in Equation 6 is unchanged. Similar reasoning can be applied to the blood contained in the left atrium and ventricle. Since this too acts as a diluent, Equation 6 is valid not only for a blood specimen collected at point A, but for a specimen withdrawn from a brachial artery, the usual sampling site.

The third assumption of the method is that the Kr^{85} in the blood leaving the alveolar limb describes a curve which, during the time of sampling, is an approximately linear function of time. This allows C_{A_K} to be expressed as shown in Equation 7.

$$C_{A_K} = Kt \quad [7]$$

where K = the slope of the line.

Substitution of Equation 7 in Equation 6 yields Equation 8.

$$\frac{C_{c_D}}{C_{c_K}} = \frac{\int_0^T C_{a_D}dt}{\frac{\dot{Q}_s}{\dot{Q}_p}\cdot\frac{C_{I_K}}{C_{I_D}}\int_0^T C_{a_D}dt + \left(1-\frac{\dot{Q}_s}{\dot{Q}_p}\right)K\int_0^T t\,dt}. \quad [8]$$

Equation 8 contains two unknown quantities: $\dot{Q}_s/\dot{Q}_p$, the fraction of the pulmonary arterial flow traversing shunts, and K, the slope of the straight line. While the presence of two unknowns renders a straightforward solution of the equation impossible, a solution can be obtained

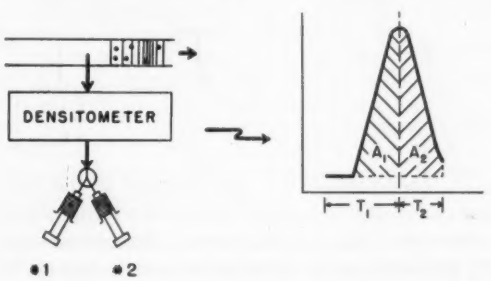

FIG. 5. SCHEMATIC DRAWING OF METHOD USED TO COLLECT TWO SAMPLES OF ARTERIAL BLOOD DURING THE FIRST CIRCULATION OF THE INDICATORS. The densitometer inscribes the dye curve directly on an oscilloscopic screen. Syringe no. 1 samples during period T_1, and syringe no. 2 during period T_2.

if arterial blood samples are collected into two syringes connected as shown in Figure 5. Syringe no. 1 samples blood through the densitometer from the time of injection to the approximate peak of the dye curve; syringe no. 2 samples from the peak of the curve to some point on the downslope before obvious recirculation of dye appears. Analyses of these two specimens for dye and Kr^{85} concentrations allow Equation 8 to be written for two different situations. Thus, for syringe no. 1

$$\left[\frac{C_{CD}}{C_{CK}}\right]_1 = \frac{\int_0^{T_1} C_{aD}dt}{\frac{\dot{Q}_s}{\dot{Q}_p}\cdot\frac{C_{IK}}{C_{ID}}\int_0^{T_1} C_{aD}dt + \left(1-\frac{\dot{Q}_s}{\dot{Q}_p}\right)K\cdot\frac{T_1^2}{2}} \quad [9]$$

and for syringe no. 2

$$\left[\frac{C_{CD}}{C_{CK}}\right]_2 = \frac{\int_{T_1}^{T_2} C_{aD}dt}{\frac{\dot{Q}_s}{\dot{Q}_p}\cdot\frac{C_{IK}}{C_{ID}}\int_{T_1}^{T_2} C_{aD}dt + \left(1-\frac{\dot{Q}_s}{\dot{Q}_p}\right)K\left(\frac{T_2^2-T_1^2}{2}\right)}. \quad [10]$$

The simultaneous solution of these two equations provides an expression for $\dot{Q}_s/\dot{Q}_p$.

$$\frac{\dot{Q}_s}{\dot{Q}_p} = \frac{\left[\frac{C_{CK}}{C_{CD}}\right]_1(T_2^2-T_1^2)\int_0^{T_1} C_{aD}dt - \left[\frac{C_{CK}}{C_{CD}}\right]_2(T_1^2)\int_{T_1}^{T_2} C_{aD}dt}{\left[\frac{C_{IK}}{C_{ID}}\right]\left[(T_2^2-T_1^2)\int_0^{T_1} C_{aD}dt - T_1^2\int_{T_1}^{T_2} C_{aD}dt\right]}. \quad [11]$$

Each integral can be evaluated by first measuring the corresponding area shown in Figure 5 and then multiplying this value by a calibrating factor, M, which converts the area to the product of concentration and time. Thus

$$\int_0^{T_1} C_{aD}dt = MA_1$$

$$\int_{T_1}^{T_2} C_{aD}dt = MA_2.$$

Since, however, an integral appears in each of the terms of the numerator and denominator of Equation 11, the necessity for calibrating the curves is obviated, and the solution of the equation is simplified by substituting the areas for the integrals which they represent.

$$\frac{\dot{Q}_s}{\dot{Q}_p} = \frac{\left[\frac{C_{CK}}{C_{CD}}\right]_1(T_2^2-T_1^2)A_1 - \left[\frac{C_{CK}}{C_{CD}}\right]_2(T_1^2)A_2}{\left[\frac{C_{IK}}{C_{ID}}\right]\left[(T_2^2-T_1^2)A_1 - T_1^2A_2\right]}. \quad [12]$$

Finally, the calculated value of $\dot{Q}_s/\dot{Q}_p$ can be used in conjunction with Equation 9 to obtain a solution for K.

$$K = \frac{\int_0^{T_1} C_{aD}dt - \left[\frac{C_{CD}}{C_{CK}}\right]_1\left[\frac{\dot{Q}_s}{\dot{Q}_p}\right]\left[\frac{C_{IK}}{C_{ID}}\right]\int_0^{T_1} C_{aD}dt}{\left[\frac{C_{CD}}{C_{CK}}\right]_1\left(1-\frac{\dot{Q}_s}{\dot{Q}_p}\right)\frac{T_1^2}{2}}. \quad [13]$$

In this equation the time integral of the concentration of dye appears only in the terms of the numerator, and, for this reason, the corresponding areas cannot be substituted as in Equation 12 unless the calibration factor M is applied.

$$K = \frac{MA_1\left\{1-\left[\frac{C_{CD}}{C_{CK}}\right]_1\left[\frac{\dot{Q}_s}{\dot{Q}_p}\right]\left[\frac{C_{IK}}{C_{ID}}\right]\right\}}{\left[\frac{C_{CD}}{C_{CK}}\right]_1\left(1-\frac{\dot{Q}_s}{\dot{Q}_p}\right)\frac{T_1^2}{2}}. \quad [14]$$

One further relation has considerable importance. This is the ratio R which defines the slope K per unit of radioactivity injected.

$$R = \frac{K}{(C_{IK})(V_I)} \quad [15]$$

where V_I = volume of dye injected. The usefulness of this ratio will be considered in the next section.

The units of the quantities included in the foregoing equations are tabulated below. If the concentrations of dye are read in plasma, the numbers must be converted to whole blood values by multiplying each by 1 − hematocrit.

Cc_K and CI_K	counts/cuvet/minute
Cc_D and CI_D	mg/cc
T_1 and T_2	seconds
A_1 and A_2	cm^2
K	(counts/cuvet/minute)/second
M	$[(mg)(sec)/L]/cm^2$
V_I	cc

Evaluation of the assumptions. As mentioned in the preceding section, the validity of the method rests on three major assumptions. The first is that the transit times through the alveolar and shunt pathways are equal. Such equality would be expected with multiple, small pulmonary arteriovenous fistulas, and, to confirm this expectation, we drew two Kr^{85} curves in each of two patients in whom such fistulas had been demonstrated by angiography. The curves were constructed by collecting sequential small anaerobic samples of blood from a manifold connected to the needle in the brachial artery. One of the curves, typical of all of them, is shown in Figure 6A. Inspection of the figure reveals that the Kr^{85} curve was approximately in phase with the dye curve; this phase correspondence suggests that in these two patients the first assumption of the method was met.

But the validity of the assumption is less certain in patients with large fistulas connecting the major pulmonary vessels near the hila of the lungs. While the construction of a Kr^{85} curve in each such patient is not practical, the validity of the assumption can still be tested in two ways. The first is the appearance of the dye curve: with a large shunt having a short transit time, a break in the curve may appear (6). The second safeguard is the ratio R defined by Equation 15. If the transit time of blood through the shunt is shorter than that through the capillaries, R will have a large negative value. While in those patients failing to fulfill the assumption the estimation of shunt-flow is not possible, the method will nevertheless indicate that such shunts exist.

The second assumption of the method, based on the belief that during the first circulation the Kr^{85} remains in the blood conveyed by shunts, is that the ratio of concentrations of dye to Kr^{85} in this blood is the same as that in the injectate. While this hypothesis could not be directly tested for shunts in the pulmonary circulation, we indirectly evaluated its validity by injecting a solution of dye and Kr^{85} into a brachial vein while collecting a blood sample from the pulmonary artery during the time of the initial circulation of the indicators. Such studies were carried out in four patients. In each, the ratio of dye to Kr^{85} in the sample was almost identical to that in the injectate.

The third assumption is that the blood leaving the alveolar limb describes an arterial concentration curve which is an approximately linear function of time. The validity of this premise is supported by two lines of evidence. The first is theoretical and comes from graphic constructions of the concentrations of Kr^{85} in blood leaving the alveolar capillaries after a bolus of the isotope has been rapidly injected into one of the great veins. With such an injection, the concentration of Kr^{85} entering the capillaries resembles either a step function or a tall triangle, and by using a modified Bohr integration, one can show that the concentration leaving the capillaries has a sigmoid relation to time. While the exact shape depends on the configuration of the bolus, the deviations from linearity of the early portions of the sigmoid curves are small.

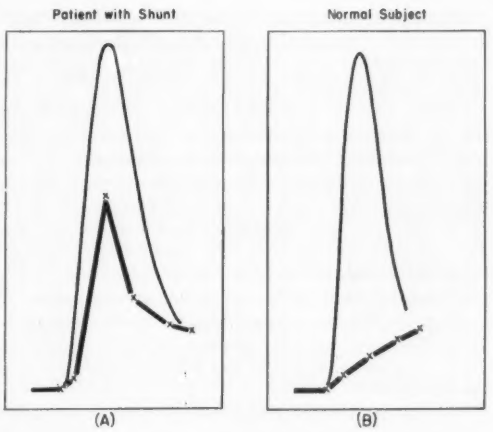

FIG. 6. COMPARISON OF TIME RELATION OF DYE (CONTINUOUS LINE) AND Kr^{85} (BROKEN LINE) CURVES IN PATIENTS WITH AND WITHOUT PULMONARY ARTERIOVENOUS SHUNTS. Time is plotted on the horizontal axis, and concentrations of the indicators on the vertical axis. For explanation of the construction of the curves, see text.

The other line of evidence comes from curves actually drawn in eight normal subjects; a typical example is shown in Figure 6B. In such subjects the pulmonary arteriovenous shunt-flow is known to be minimal (7–9), and, as expected, the curves approximated straight lines.

A fourth assumption, implied but not previously mentioned, is that the method is insensitive to the other factors that can lower the saturation of the arterial blood. These factors include inadequate ventilation, disturbed ventilation-perfusion relationships, impaired diffusion, and abnormal hemoglobin. None of these factors directly affects the dye curve, and none influences the Kr^{85} curve to any great extent. For instance, inadequate ventilation and disturbed ventilation-perfusion ratios are of less importance during the brief initial passage of the indicators than are the volumes of air contained in the alveoli contacted by the Kr^{85}-carrying blood. To investigate the effect of different lung volumes, we drew successive curves in six normal subjects while they held their breaths at different fractions of the vital capacity. Whereas the slopes of the Kr^{85} curves decreased with increasing lung volumes, the linearity of the curves was not affected.

Although a reduced diffusing capacity may influence the arterial Kr^{85} concentration, a moderately thickened membrane should be of only small consequence since inert gases reach equilibrium so rapidly.

Finally, abnormal hemoblobin can probably be discounted because Kr^{85} is believed to be carried in simple physical solution in both the plasma and the cells.

Procedure in patients. The details of preparing the solutions for injection have been published previously (5). In the present study, the concentrations of dye and Kr^{85} were adjusted so that each cubic centimeter of dye contained approximately 50 μc of radioactivity.

All patients were studied in the fasting, basal state. A small polyethylene catheter was advanced through an antecubital vein until the catheter tip lay in the axillary vein. A Cournand needle was inserted into the right brachial artery.

The experimental procedure was started after the patient had rested for 15 minutes with the catheter and needle in place. The patient breathed through a mouthpiece which connected him to an open breathing circuit. After 5 minutes of quiet breathing, 3 cc of the dye and Kr^{85} mixture was rapidly injected into the catheter while arterial blood was withdrawn at a constant rate through a Colson densitometer into the syringe designated no. 1 in Figure 5. Near the peak of the curve, the stopcock was turned and syringe no. 2 continued the sampling to a point on the downslope short of obvious recirculation of dye. Each of the collected samples was thoroughly mixed, then divided into two parts: one was used to determine the concentration of dye in plasma, the other was used to assay the radioactivity in hemolyzed blood.

TABLE I

Measured values of arterial oxygen saturation (S_{aO_2}) and calculated values of shunt-flow ($\dot{Q}_s/\dot{Q}_p$)

Subject	Diagnosis	Sa_{O_2}	$\dot{Q}_s/\dot{Q}_p$
		%	%
	Group I: Control subjects		
H.Y.		97	<1
J.M.		96	<1
W.S.		95	<1
E.H.		95	<1
A.G.		95	<1
J.S.		95	<1
J.R.		94	<1
N.R.		94	<1
	Group II: Patients with pulmonary emphysema		
A.S.		92	<1
J.W.		92	<1
L.B.		91	<1
J.G.		90	<1
W.D.		89	<1
S.V.		86	<1
M.W.		85	<1
H.G.		81	2
	Group III: Patients with pulmonary infections		
T.D.	Chronic bronchitis	91	<1
J.Q.	Chronic bronchitis	88	2
B.B.	Bronchiectasis	90	<1
W.P.	Bronchiectasis	93	<1
J.B.	Unresolved pneumonia	92	<1
C.T.	Unresolved pneumonia	87	<1
E.Mc.	Advanced pulmonary tuberculosis	91	<1
E.D.	Advanced pulmonary tuberculosis	90	<1
W.B.	Advanced pulmonary tuberculosis	89	<1
H.P.	Advanced pulmonary tuberculosis	78	2
A.B.	Advanced pulmonary tuberculosis	77	<1
L.P.	Advanced pulmonary tuberculosis and alcoholic cirrhosis of the liver	88	8
	Group IV: Patients with alcoholic cirrhosis of the liver		
J.R.		97	<1
P.K.		95	<1
P.B.		95	<1
C.K.		94	<1
J.K.		94	<1
A.M.		92	<1
W.K.		90	<1
F.M.		90	3
R.W.		90	2
W.D.		89	<1
J.P.		89	2
A.		88	<1
A.A.		83	<1
F.J.		79	<1
	Group V: Patients with Weber-Rendu-Osler's disease		
M.S.		94	12
J.L.		91	9
J.P.		72	57

The concentration of radioactivity in each of the blood specimens was measured by the method of Lassen and Munck (10). For this measurement the hemolyzed sample was transferred to an air-tight, disc-shaped cuvet of 1.5 cc volume, and the radioactivity emitted from each face was counted with a Geiger-Mueller tube. These counts, made simultaneously, were summed by one scaler. Each count was continued for a sufficiently long period to insure that the statistical error was less than 5 per cent.

Dye concentrations were read in plasma, using a Beckman Model DU spectrophotometer.

Arterial blood oxygen saturations were determined by the method of Van Slyke and Neil.

Patient material. Five groups of patients were studied. The arterial saturation of each patient is recorded in Table I.

The first group comprised 8 men and women who were either healthy or who had minimal apical tuberculosis. These subjects served as controls.

The second group included 8 patients with clinical, roentgenological and physiological stigmata of advanced pulmonary emphysema.

The third group was composed of 12 men and women with pulmonary infections, including chronic bronchitis, bronchiectasis, unresolved pneumonia, and advanced pulmonary tuberculosis. Many of these patients had X-ray evidence of widespread pulmonary fibrosis.

The fourth group embraced 14 patients with alcoholic cirrhosis of the liver. Although none had evidence of heart or lung disease, 9 had arterial saturations below 94 per cent.

The fifth group was made up of 1 man and 2 women with Weber-Rendu-Osler's disease. Each had a low arterial saturation, and each had had one or more pulmonary arteriovenous fistulas demonstrated by angiography.

RESULTS

The results are presented in Table I. The shunts calculated in the normal subjects were in some instances slightly negative due, presumably, to the fact that the Kr^{85} curves are sigmoids rather than absolutely straight lines. In no subject, however, was the magnitude of the calculated shunt greater than 1 per cent of the flow through the pulmonary artery.

Seven of the patients with emphysema had values which were no different from those calculated in the normals; the eighth (H. G.) had an estimated shunt-flow of 2 per cent.

Nine of the patients with pulmonary infections had values of shunt-flows within the normal range, while three (J. Q., H. P. and L. P.) had values of 2, 2 and 8 per cent, respectively.

Eleven of the patients with cirrhosis had shunt-flows of less than 1 per cent, two had 2 per cent, and one had 3 per cent.

By way of contrast, the patients with Weber-Rendu-Osler's disease had shunts between 9 and 57 per cent. Thus, only in these patients did shunts appear to account for the levels of saturation in the arterial blood.

The ratio R was calculated for each patient studied. With a single exception, there was no difference between the values found in the normal subjects and those calculated in the patient groups. The average for the normals was 12.2 with a standard deviation of 7.7. Further, these values agreed with the average value of 12.6 ± 5.7 obtained in eight normal subjects in whom the curves of Kr^{85} concentration were actually drawn. The only exception was a case of Weber-Rendu-Osler's disease with large anastomoses near the hila of the lungs. The value of R in this patient was $-1{,}500$, suggesting that the transit times through the shunts were shorter than those through the capillaries. The appearance of the dye curve supported this belief; instead of having a smooth contour, the curve showed two distinct peaks.

DISCUSSION

Advantages and disadvantages of the method. The major advantage of the method is its specificity for detecting the presence of pulmonary arteriovenous shunts (11). Previous techniques have not clearly distinguished between the flow through pulmonary arteriovenous connections and other sources of venous blood. Recently, Jose and Milnor (12) have used the Valsalva maneuver to demonstrate the presence of shunting, but have carefully emphasized that a shunt can be mimicked by severe ventilation-perfusion disturbances. Recently also Braunwald, Long and Morrow (13), as well as Long, Waldhausen, Cornell and Sanders (14), have used intravenous injections of Kr^{85} alone to demonstrate that pulmonary arteriovenous shunts exist.

The second advantage of the method resides in the fact that it is not influenced by the level of the arterial saturation, nor does it require that this level be changed. This point is of particular importance in patients with emphysema in whom the administration of oxygen may alter the distribution of blood flow in the lungs.

The chief disadvantage stems from the assumption that the curves of Kr^{85} in blood perfusing alveoli are linear. This premise imposes the greatest handicap when shunts carry only a small proportion of the pulmonary arterial blood flow, and, for this reason, the method is not suitable for estimating the shunt-flow existing in normal man. That the estimates of larger shunt-flows are more reliable is suggested by the results in the patients with Weber-Rendu-Osler's disease. In these patients, the calculated shunts were compatible with the levels of saturation measured in the arterial blood.

A second disadvantage exists because Kr^{85} and oxygen have different solubilities in the pulmonary membrane. Hence, a vessel might serve as a shunt for oxygen and not for Kr^{85}.

Results in patients with pulmonary diseases. The fact that shunts were not found in the patients with emphysema or with pulmonary infections does not rule out the possibility that in some patients with these diseases such shunts exist. Emphysema in particular is characterized by its protean manifestations, and it is conceivable that shunts sometimes occur in this complex disease. However, others have published data which make the invariable presence of sizable shunts unlikely (15). In addition, recent studies have provided evidence that the arterial unsaturation in patients with this disorder can be satisfactorily explained by ventilation-perfusion disturbances alone (16–18).

Without question, some patients with emphysema fail to achieve the expected arterial oxygen content when they breathe oxygen, a phenomenon generally attributed to the shunting of venous blood. An alternative explanation may lie in the behavior of those alveoli which are grossly underventilated; these have tensions of oxygen so close to that of the venous blood entering the capillaries that the exchange of gas is

TABLE II

Comparison of average ratios of mixed venous ($C_{\bar{v}}$) and brachial arterial (C_a) blood concentrations of Kr^{85} when the isotope was infused first into the duodenum and then into the right atrium

	$C_{\bar{v}}/C_a$ during duodenal infusion	$C_{\bar{v}}/C_a$ during right atrial infusion
4 Normal subjects	11.9	11.5
5 Patients with cirrhosis	5.5	14.0

minimized to the point where these capillaries serve as pulmonary arteriovenous shunts. But the reduced ventilation should not prevent the escape of Kr^{85} during its initial passage through these vessels. Thus, the oxygen and Kr^{85} methods probably detect different anatomic pathways; the former includes all channels in which gas exchange is minimal, while the latter detects that fraction of these channels deprived of contact with alveolar gas.

Results in patients with alcoholic cirrhosis of the liver. Although the fact that patients with cirrhosis are sometimes cyanotic has been recognized for almost a century (19), the cause of the cyanosis is not yet known. In 1938 Keys and Snell (20) suggested that the fundamental affinity of hemoglobin for oxygen is reduced in cirrhosis, but Jandl (21) later questioned this view. More recently, Abelmann and his co-workers (22) and Heinemann, Emirgil and Mijnssen (23) have shown that an augmented venous admixture is at least partially at fault. In this connection, Rydell and Hoffbauer (24) demonstrated multiple, small pulmonary arteriovenous fistulas in a young man with juvenile cirrhosis, and suggested that shunting through these channels accounted for the cyanosis which existed during life. While this observation has led others to invoke the same mechanism in patients with alcoholic disease of the liver, the results of the present study appear to rule out the possibility that direct connections between the pulmonary artery and the pulmonary veins invariably account for the lowered saturation of the arterial blood.

An alternative explanation, previously implied by McIndoe (25) and by Schoenmackers and Vieten (26), has recently been investigated by Calabresi and Abelmann (27). From injection studies performed at autopsy, these last investigators adduced evidence that patients with Laennec's cirrhosis have vascular anastomoses which connect the portal circulation to the pulmonary veins. To investigate whether these channels carry blood in living humans, we studied five patients with alcoholic cirrhosis and four normal subjects, using a technique which entailed catheterization of the pulmonary artery, cannulation of the brachial artery, and installation of a Levine tube in the duodenum. Kr^{85} was first introduced into the portal circulation by infusing a solution of this gas into the duodenum through the Levine tube. Since preliminary observations had indicated that this procedure produced steady concentrations of Kr^{85} in the arterial and mixed venous blood streams for periods of from 5 to 9 minutes, blood samples were simultaneously collected from the pulmonary and brachial arteries at 1-minute intervals during this time. The patient then rested quietly until all of the Kr^{85} had been eliminated via his expired gas. In a second run, an infusion of Kr^{85} was directed into the right atrium and samples of arterial and venous blood were again obtained. The concentrations of Kr^{85} in the collected blood specimens were measured. The results are summarized in Table II.

In the four normal subjects, the ratios of venous to arterial concentrations were approximately the same for each route of administration. By way of contrast, the five cirrhotic patients had smaller ratios when the Kr^{85} was introduced into the duodenum than when it was infused into the right atrium. Since none of the patients manifested a pulmonary arteriovenous shunt when tested by the single injection method, the smaller ratios suggest that blood from the portal veins entered the pulmonary vessels beyond the capillary bed. While these observations support the belief that portal-mediastinal-pulmonary connections contributed to the unsaturation, they do not provide indisputable evidence that these connections were the sole cause. In the first place, the method will not allow a quantitative estimate of the blood flowing through these channels. Secondly, on the basis of the blood oxygen saturation found in the portal circulation by Smythe, Fitzpatrick and Blakemore (28), the volume of anastomotic flow necessary to lower the arterial saturation to the level seen in some patients with cirrhosis would have to be a

remarkably large fraction of the flow delivered to the entire splanchnic bed.

SUMMARY

The intravenous injection of Kr^{85} dissolved in T-1824 dye provides a method for estimating the fraction of the pulmonary arterial blood flow perfusing arteriovenous shunts. As expected, large shunts were present in patients with Weber-Rendu-Osler's disease who exhibited arterial blood oxygen unsaturation. By way of contrast, no sizable shunts were evident in 8 patients with pulmonary emphysema or in 12 patients with pulmonary infections. Further, only small shunts were found in 14 patients with Laennec's cirrhosis, even though 9 of these patients had arterial saturations below 94 per cent. Additional studies on 5 of these patients suggested that vascular anastomoses connected the portal bed with the post-capillary segments of the pulmonary vessels. These studies did not reveal the size of the flow through the anastomoses, nor whether this flow fully accounted for the reduced saturation of the arterial blood.

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ADRENAL STEROID INHIBITION OF VASOPRESSIN RELEASE FROM THE NEUROHYPOPHYSIS OF NORMAL SUBJECTS AND PATIENTS WITH ADDISON'S DISEASE *

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There is a large body of evidence to indicate that adrenal steroids decrease distal renal tubular reabsorption of water. A popular view is that this effect may be due to antagonism of the action of vasopressin on the renal tubules (1), but others (2) have postulated that corticoids may exert a direct effect on permeability of the renal tubular cells to water. Recent studies in patients with combined anterior and posterior pituitary insufficiency (3) suggested that adrenal steroids increase free water excretion by suppressing release of vasopressin from hypothalamic portions of the neurohypophysis. Further investigation of this preliminary hypothesis in normal subjects and in patients with Addison's disease forms the basis of this report.[1]

MATERIALS AND METHODS

Studies of water excretion were performed in 8 individuals with apparently normal adrenal and pituitary function and in 3 patients with Addison's disease. Fluids were withheld after 10 p.m. and all subjects abstained from smoking on the morning of study. Each subject was given a light breakfast including 90 ml of fruit juice at 7 a.m., followed by 1.0 to 1.5 L tap water by mouth at about 8 a.m. Three of the normal subjects (depicted in Figures 1 and 3) were laboratory personnel who continued their duties throughout the study and collected voided urine specimens at 15-minute intervals until diuresis subsided.

The remaining normal subjects and the 3 patients were recumbent during study and maintained in a constant state of hydration by administration of tap water orally or of 5 per cent dextrose and water intravenously at a rate equal to urine flow. Urine was collected at 15 to 30 minute intervals by spontaneous voiding in 5 subjects and through an indwelling urethral catheter in 3.

The effect of adrenal steroids on the antidiuretic response to nicotine was evaluated in all subjects. Nicotine was administered by vigorous inhalation of cigarette smoke in 3 normal subjects and by intravenous infusion of solutions of nicotine salicylate (8) or nicotine bitartrate [2] in the remainder. Some subjects subsequently received Pitressin [3] (vasopressin) and 3 per cent

TABLE I

Effect of nicotine on renal function in normal subject (P.B.) and the action of adrenal steroids thereon *

Study	U_{osm}†	C_{H_2O}†	C_{osm}‡	C_{cr}‡
	mOsm/kg	*ml/min*	*ml/min*	*ml/min*
Controls				
I§	83	7.4	3.2	132
II§	77	8.7	3.0	127
Nic. 3.5‖	214	1.7	5.9	133
4.0	243	0.4	3.9	130
5.0	488	−1.4	3.8	115
F1-F¶				
§	44	15.3	2.2	131
Nic. 3.0‖	45	13.3	3.1	129
4.5	178	2.1	3.2	120
DCA**				
§	77	8.8	3.1	123
Nic. 3.0‖	75	10.0	3.7	120
4.0	112	8.6	4.2	119
4.5	86	9.0	3.6	115

* Constant water load of 1.0 L maintained throughout each study.

† Minimum U_{osm} and maximum C_{H_2O} during water diuresis; maximum U_{osm} and minimum C_{H_2O} following administration of nicotine.

‡ Mean values for entire periods of water diuresis and of nicotine antidiuresis.

§ Values observed during water diuresis.

‖ Milligrams of nicotine i.v.

¶ 1.0 mg fluorocortisol by mouth every 6 hours for 2 days prior to study.

** 10 mg DCA i.m. 12 hours before and 5 mg 2 hours prior to hydration.

* Supported in part by grants from the National Heart Institute (H2106 and H2850), the Louisiana Heart Association, and the Upjohn Company.

† Research assistant on Grant H2106, National Heart Institute, Bethesda, Md.

[1] Portions of these studies have been reported previously (4–7).

[2] Supplied through the kindness of Dr. R. C. Hockett, Tobacco Industry Research Committee, New York, N. Y.

[3] Supplied through the courtesy of Dr. J. E. Gajewski, Parke, Davis & Co., Detroit, Mich.

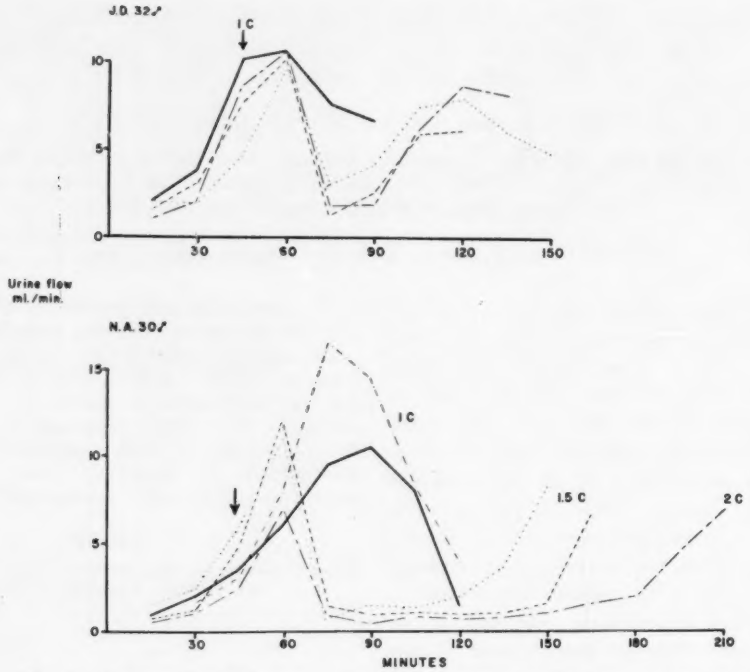

FIG. 1. ANTIDIURETIC RESPONSE TO CIGARETTE SMOKING IN TWO NORMAL SUBJECTS. 1.0 L water imbibed at zero time. Both subjects smoked the designated number of cigarettes (C) at 45 minutes, as indicated by the arrows. The solid line depicts the curve of water diuresis in one control study.

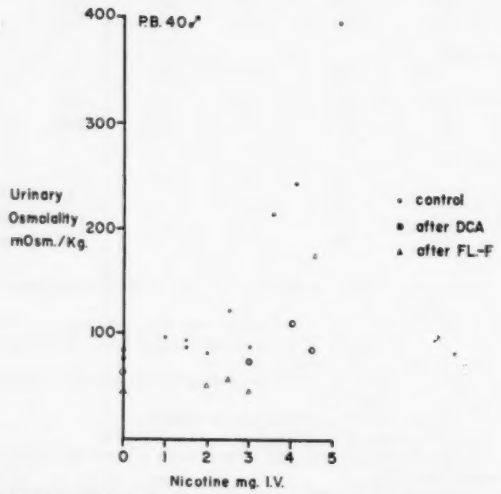

FIG. 2. EFFECT OF ADRENAL STEROIDS ON ANTIDIURETIC RESPONSE TO NICOTINE IN A NORMAL SUBJECT. Studies performed during sustained hydration of 1.0 L. Data obtained from two separate control studies and one study each with intramuscular desoxycorticosterone acetate (DCA) and oral fluorocortisol (Fl-F). Points at zero on the horizontal scale represent minimum U_{osm} observed prior to administration of nicotine intravenously.

NaCl solution intravenously according to a described technic (8).

Urinary and serum concentrations of Na and K were measured with a Baird flame photometer, total solute concentration by freezing point depression with a Fiske osmometer, and creatinine by a modification of the Jaffé reaction (9). Free water clearance (C_{H_2O}) and osmolal clearance (C_{osm}) were calculated using the formulas of Wesson and Anslow (10). The renal tubular action of endogenous and exogenous vasopressin was estimated from acute changes in urinary osmolality (U_{osm}) and C_{H_2O} (8). The duration of the antidiuretic response (i.e., interval from injection of vasopressin or onset of endogenous vasopressin antidiuresis to return of C_{H_2O} and U_{osm} to pre-injection levels) was also utilized as an index of the renal response to vasopressin and of the magnitude of endogenous vasopressin release.[4]

The Addisonian patients had been treated with 25 mg of cortisone acetate by mouth daily, which was with-

[4] The duration of antidiuresis has been shown to bear a linear relationship to the log-dose of vasopressin (11), which provides a means of quantitating vasopressin release and assessing the renal effects of the hormone.

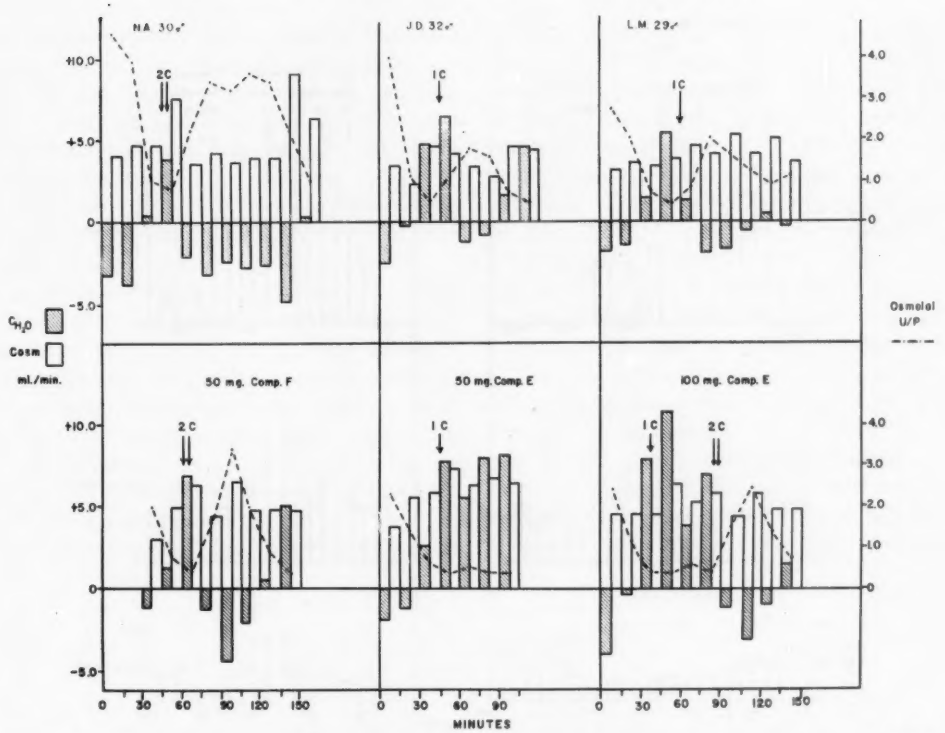

FIG. 3. EFFECT OF ADRENAL STEROIDS ON ANTIDIURETIC RESPONSE TO NICOTINE IN NORMAL SUBJECTS. 1.0 L water imbibed at zero time. Steroids administered orally 2 hours prior to study.

held as specified. Two patients required monthly injections of desoxycorticosterone trimethylacetate in doses of 37.5 and 50 mg, respectively, to maintain normal salt balance, but the third patient was maintained in good health with cortisone and a liberal salt intake only.

RESULTS

1. Reproducibility of the antidiuretic effect of nicotine. The antidiuretic effect of nicotine (cigarette smoking) was found to be fairly reproducible in two normal subjects (Figure 1) and, in one subject so studied, the duration of antidiuresis appeared to be related to the dose of nicotine. A third normal subject received repeated injections of nicotine intravenously at increasing dose levels in two control studies performed during constant water diuresis (control studies, Figure 2 and Table I). Although this subject was a "heavy" smoker and had an unusually high threshold for nicotine antidiuresis, the magnitude of the antidiuretic response showed a close relationship to the administered dose of nicotine. A slight to moderate increase in C_{osm} and no change in creatinine clearance, (C_{cr}), except for a slight fall with 5.0 mg nicotine, were observed during the periods of antidiuresis (Table I).

Approximately equal antidiuretic responses to repeated injections of 1.5 mg of nicotine were observed in one patient with Addison's disease, and 5 mg Regitine (phentolamine) administered intravenously several minutes prior to one injection, did not alter the antidiuretic effect in any way.

2. Effect of glucocorticoids in normal subjects. Cortisone, cortisol and prednisolone administered orally 1 to 2 hours prior to standard hydration (Figures 3 and 4) or intravenously during sustained diuresis (Figures 5 and 6) resulted in suppression or complete inhibition of the antidiuretic response to previously effective doses of nicotine in each of six normal subjects. The antidiuretic effects of vasopressin and hypertonic saline were not similarly influenced by these steroids (Figures 5 and 6).

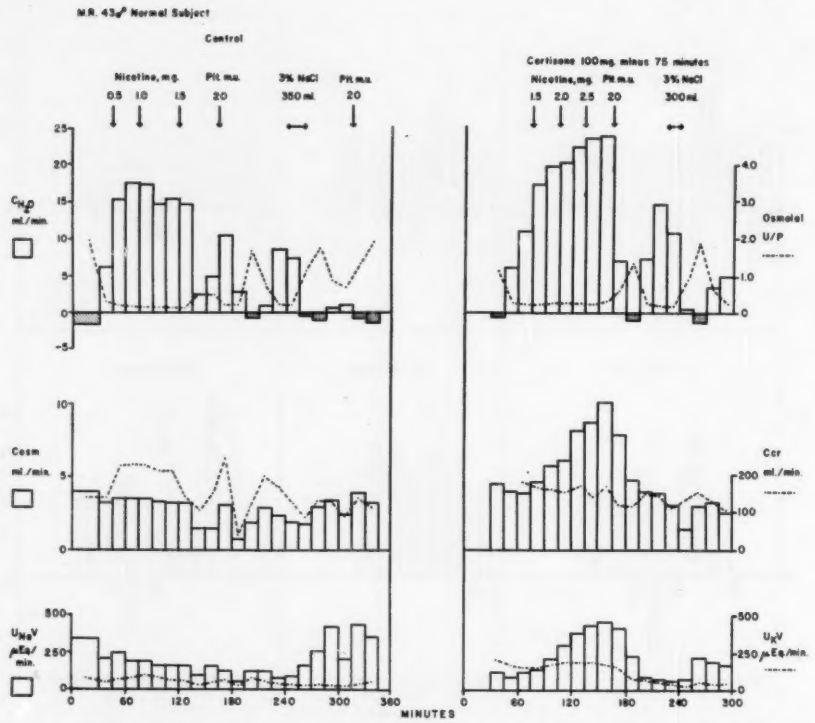

FIG. 4. EFFECT OF CORTISONE ON NEUROHYPOPHYSEAL FUNCTION. Sustained hydration of 1.5 L; Pit.mU. = milliunits vasopressin (Pitressin) i.v.

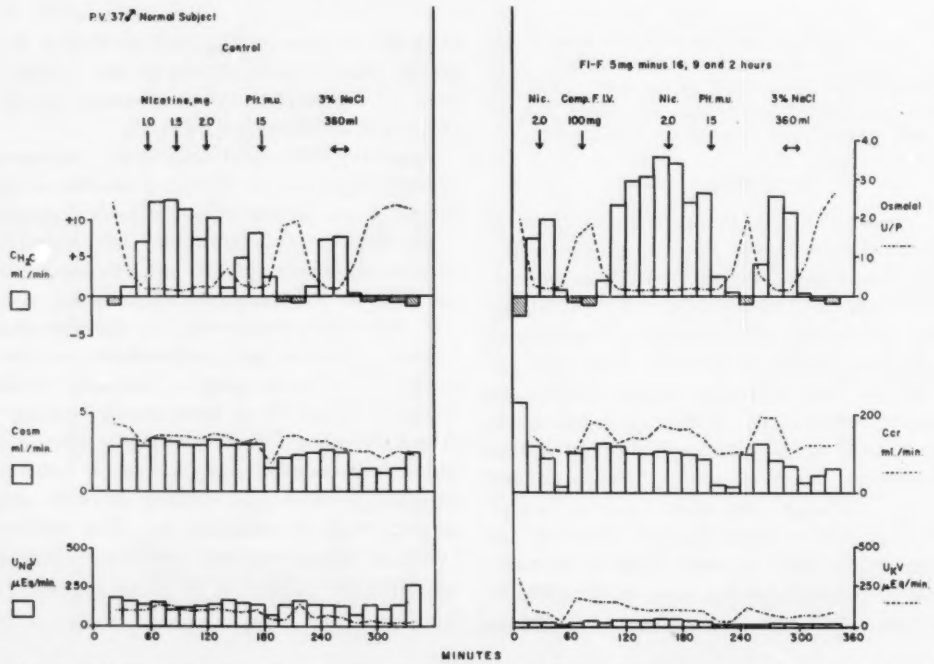

FIG. 5. EFFECT OF ADRENAL STEROIDS ON NEUROHYPOPHYSEAL FUNCTION. Sustained hydration of 1.3 L.

The inhibitory effect of glucocorticoids on nicotine antidiuresis was frequently preceded by and associated with increases in peak C_{H_2O} and concomitant decreases in minimum U_{osm}, indicative of enhanced water diuresis despite conditions of constant hydration.

There were only slight changes observed in C_{osm} during these same periods in three subjects (Figure 3), but in one (M.R., Figure 4), a marked increase in C_{osm} accompanied the profuse free water diuresis. In the subjects given steroids intravenously (Figures 5 and 6), the depressed rates of Na and total solute excretion resulting from previous Na-retaining steroid therapy were not altered following glucocorticoid administration, yet enhanced C_{H_2O} was observed in one (Figure 5) and inhibition of nicotine antidiuresis was clearly apparent in both.

Acute decreases in C_{cr} were observed during the antidiuretic responses to nicotine, vasopressin and hypertonic saline in several control studies and were less apparent for similar periods in steroid studies. Since sudden changes in urinary flow result in unavoidable errors in calculation of clearances, mean C_{cr} values were determined for each period to include the time from onset of oliguria to the return of water diuresis. These values were compared with the mean C_{cr} observed during water diuresis in each study as well as for similar periods in control and steroid studies.

Nicotine produced an average change in mean C_{cr} of -2 per cent in four control studies and $+1$ per cent in four steroid studies. Vasopressin resulted in an average fall in mean C_{cr} of 7 and 10 per cent, and hypertonic saline a decrease of 24 and 26 per cent, respectively, in two control and in two steroid studies, but the observations were too few for valid conclusions.

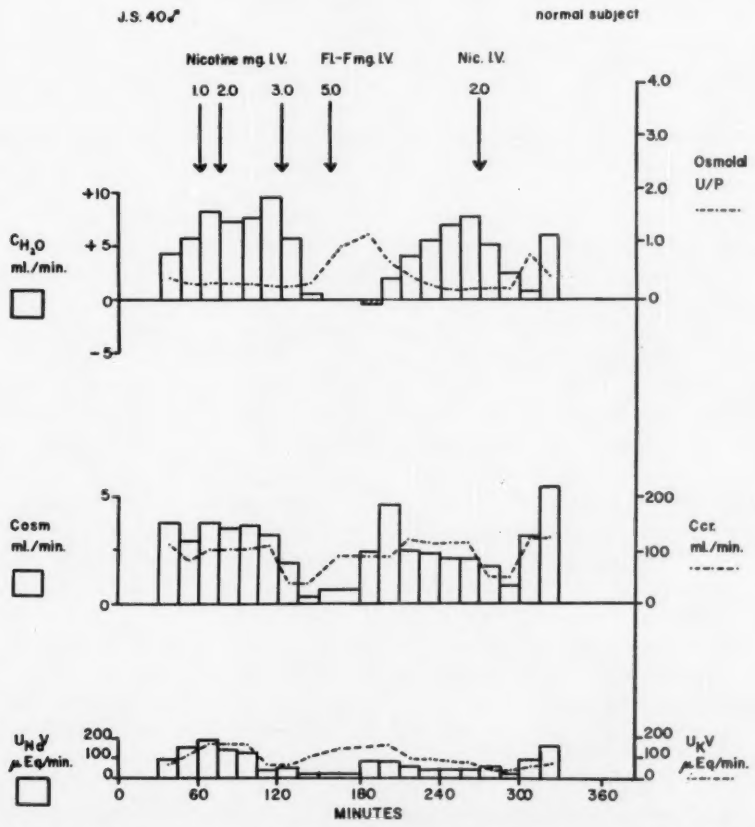

FIG. 6. EFFECT OF FLUOROCORTISOL ON ANTIDIURETIC RESPONSE TO NICOTINE. Sustained hydration of 1.0 L.

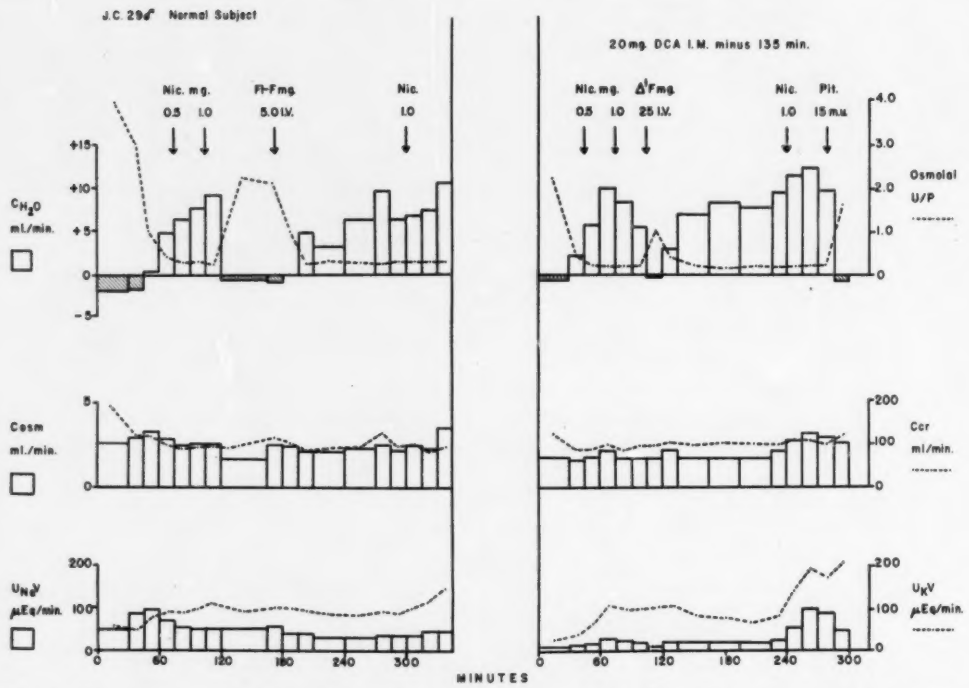

FIG. 7. EFFECT OF ADRENAL STEROIDS ON ANTIDIURETIC RESPONSE TO NICOTINE. Sustained hydration of 1.0 L; Δ^1F = prednisolone.

Steroid therapy by itself resulted in an average increase in C_{cr} of only 3 per cent in the four subjects studied.

3. Effect of fluorocortisol (Fl-F) in normal subjects. Two subjects showed depression of the antidiuretic effect of nicotine with administration of Fl-F (Table I, Figures 2 and 7). However, this steroid enhanced the antidiuretic response to a standard dose of nicotine in a third subject (Figure 5) and actually decreased the effective antidiuretic dose of nicotine in another (Figure 6). In the latter subject, the toxic effects of nicotine on the central nervous system (dizziness, nausea) were also markedly accentuated.

Marked Na retention, associated with a decrease in minimum U_{osm} during diuresis was observed in three of these subjects. A distinct increase in C_{H_2O} was apparent, however, only in the one subject who showed the most pronounced suppression of nicotine effect (Table I).

Acute changes in C_{cr} were also evident with administration of nicotine in several of these studies. Calculation of mean C_{cr} values as previously described, however, revealed average decreases of only 6 and 4 per cent, respectively, for control and steroid studies, and the steroid itself produced only a slight rise of 7 per cent in mean C_{cr} in comparison to control diuresis values.

4. Effects of DCA in normal subjects. Large doses of DCA administered prior to constant hydration resulted in marked Na retention and suppression of nicotine antidiuresis in two subjects (Table I, Figures 2 and 7). Although the onset of water diuresis was more rapid in both, C_{H_2O} was increased only in Subject J.C., who also showed a definite fall in total solute excretion. Changes in mean C_{cr} during nicotine antidiuresis equalled -11 and -14 per cent in the control studies and $+2$ and -6 per cent in the DCA studies.

5. Effect of glucocorticoids in Addison's disease. Control studies performed in three patients with Addison's disease off cortisone therapy for periods of 1 to 3 days revealed relatively normal patterns of water diuresis in two patients (Figures 8, 9 and 10) and marked depression of diu-

resis in a third (Figure 11). One of the former patients evidenced a normal antidiuretic response to nicotine (Figure 8), but the other showed increased sensitivity of the neurohypophysis to stimuli, with definite "neural" stimulation of vasopressin release apparent with as little as 0.5 mg nicotine (Figure 9) as well as with a relatively trivial painful stimulus elicited by manipulation of an indwelling intravenous needle (Figure 10). Sensitivity of the osmotic control of vasopressin release was also apparent in this patient, as evidenced by a substantial antidiuretic response with administration of as little as 5 ml of 3 per cent NaCl per kg (Figure 10); however, the antidiuretic effect of vasopressin was found to be within normal limits (Figure 9). The patient with the depressed diuresis showed a prolonged antidiuretic response to 0.5 mg nicotine associated with a rise in U_{osm} only to the isosmotic level.

Twenty-five to 50 mg cortisone administered by mouth within several hours of hydration resulted in greater urinary hypotonicity, enhanced free water diuresis, and depression of the antidiuretic effect of nicotine in each patient (Figures 8, 11 and 12). Intravenous administration of 25 mg prednisolone, a glucocorticoid four to five times more potent than cortisone, resulted in much higher levels of water diuresis and greater suppression of the antidiuretic effect of nicotine than had been observed with similar doses of oral cortisone in two patients (Figures 8 and 11). The antidiuretic effects of vasopressin and hypertonic saline were essentially unaltered by cortisone therapy in one patient so studied (Figure 12).

A substantial increase in Na and total solute excretion accompanied the enhanced water diuresis observed during cortisone therapy in two patients (Figures 8 and 12). Mean C_{cr} showed a substantial decrease of 35, 39 and 48 per cent with administration of nicotine, vasopressin and hypertonic saline, respectively, during the control studies in Patient M.R. (Figures 9 and 10). Less pronounced decreases of 14, 26 and 33 per

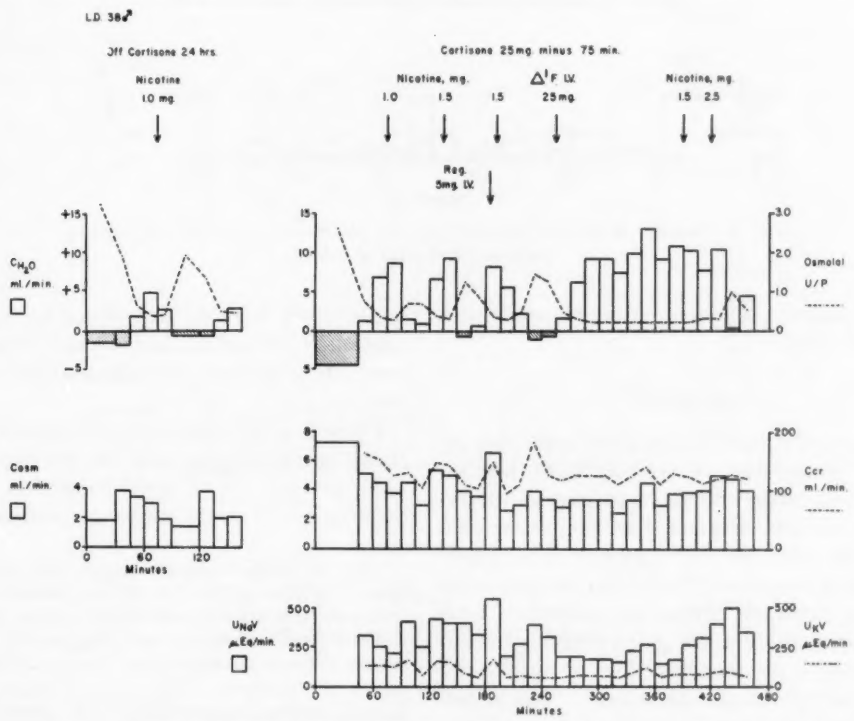

FIG. 8. EFFECT OF ADRENAL STEROIDS ON NICOTINE ANTIDIURESIS IN ADDISON'S DISEASE. Sustained hydration of 1.0 L; Reg. = phentolamine (Regitine) i.v.

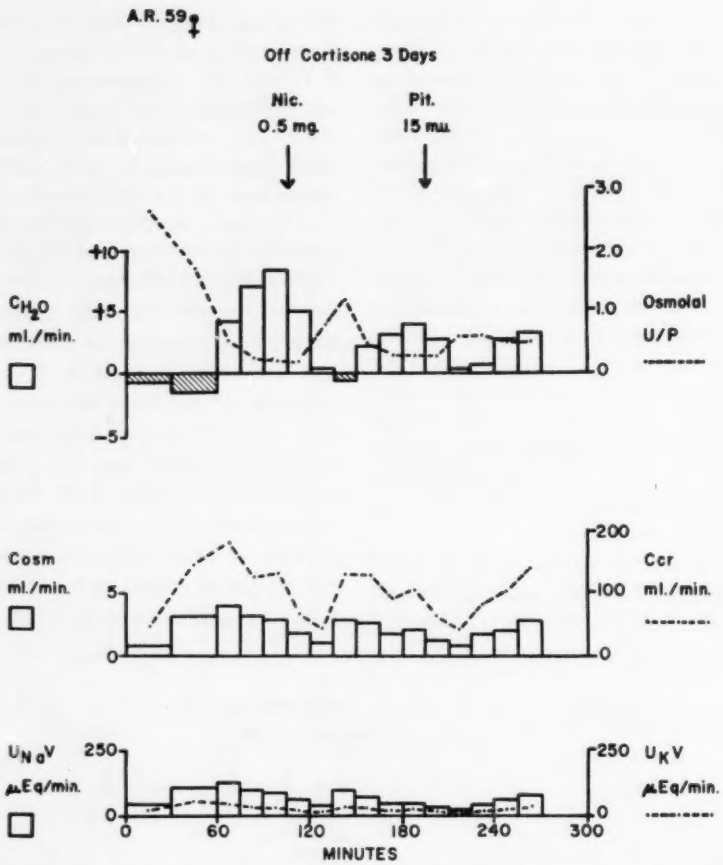

FIG. 9. ANTIDIURETIC EFFECT OF NICOTINE AND VASOPRESSIN IN ADDISON'S DISEASE. Sustained hydration of 1.2 L.

cent, respectively, appeared during the study with cortisone (Figure 12).

DISCUSSION

These studies have shown fairly consistent effects of cortisol-like steroids on water metabolism in man; these include shortening of the delay time in onset of water diuresis, greater dilution of the urine, and increased free water excretion both in normal and Addisonian subjects studied under conditions of standard hydration (Table II). Simultaneously, the glucocorticoids consistently elevated the threshold dose of nicotine for stimulation of vasopressin release. The neurohypophyseal response to induced hypertonicity and the antidiuretic effect of exogenous vasopressin, however, were not suppressed by steroids or masked by the sodium and solute diuresis which occasionally occurred with glucocorticoid administration.

There is good evidence that nicotine stimulates release of vasopressin from the neurohypophysis (12), probably by an acetylcholine-like action of the drug (13) [5] on the supraoptic neurones of the

[5] The fact that vasopressin release with nicotine was apparent in three patients with primary adrenocortical insufficiency, who often demonstrate atrophy or destruction of the adrenal medullae as well, suggests that the antidiuretic action of nicotine in man is not mediated through release of epinephrine. The failure of phentolamine to suppress nicotine antidiuresis in one patient was evidence against an effect of circulating sympathomimetic amines on neurohypophyseal secretion.

hypothalamus, resulting in release of vasopressin stored in the posterior lobe of the pituitary body into the systemic circulation.

Hypertonicity of the body fluids also promotes release of vasopressin (14), but there are some studies in man (15) and dog (16), and theoretical considerations (17) to suggest that the osmoregulatory response may not be mediated through the same pathways to the neurohypophysis as nicotine.

The results of the present study are compatible with the hypothesis that glucocorticoids suppress the response of the supraoptic neurones of the hypothalamus to neural stimuli without altering the osmotic control of vasopressin release. Corroborative evidence for inhibitory actions of glucocorticoids on hypothalamic neurosecretion is their rapid effect on ACTH release (18), which may be through suppression of secretion of corticotropin-releasing factor from the hypothalamus (19).

Since preliminary publication of portions of these data (4–7), Gaunt, Lloyd and Chart (20) reported an increase in neurosecretory material in the posterior pituitaries of rats treated with cortisol, compatible with a suppressive action of

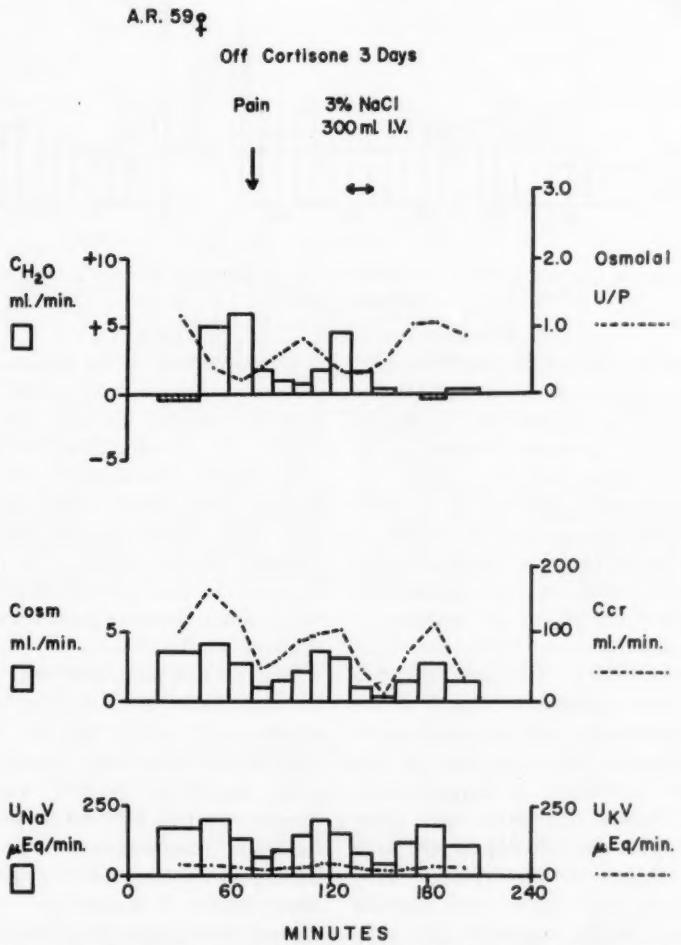

FIG. 10. EFFECT OF PAIN AND HYPERTONIC SALINE ON VASOPRESSIN RELEASE IN ADDISON'S DISEASE. Sustained hydration of 1.2 L.

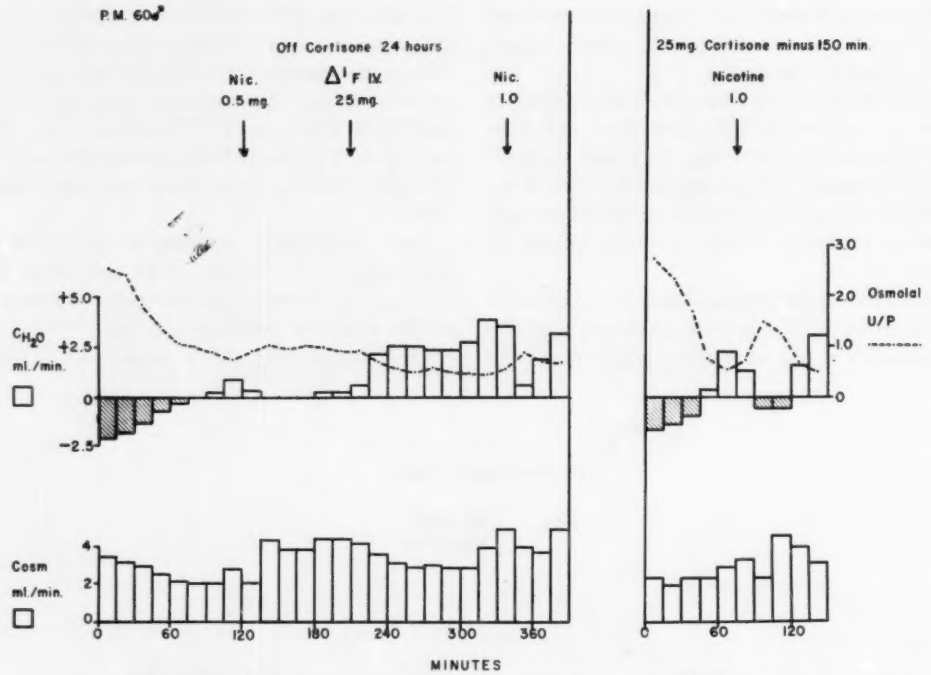

FIG. 11. EFFECT OF ADRENAL STEROIDS ON NICOTINE ANTIDIURESIS IN ADDISON'S DISEASE. Sustained hydration of 1.0 L.

this steroid on vasopressin release, and McCann, Fruit and Fulford (21), and de Wied and Mirsky (22) have shown that glucocorticoids rapidly block the antidiuretic response to nicotine and to other stressful stimuli in the rat. These results provide experimental evidence from other laboratories to corroborate our findings in man.

These studies suggest a possible explanation for the abnormal water diuresis of adrenocortical insufficiency which is related to an antidiuretic mechanism not under the control of extracellular fluid osmotic pressure (23). The neurohypophysis probably is overly sensitive to stimuli in the absence of glucocorticoids, and increased sensitivity to the antidiuretic effects of nicotine has been demonstrated previously in hypophysectomized patients (24) and in patients with spontaneous pan-hypopituitarism (3, 25) as well as in the Addisonian subjects in this report. Many studies have shown that neural and vascular (ischemic) stimuli readily supersede the relatively weak inhibitory effect of plasma hypotonicity on vasopressin release (17). An inhibitory effect of glucocorticoids on the responsiveness of the supraoptic nuclei to such stimuli may normalize neurohypophyseal function and permit adequate suppression of vasopressin release from the posterior lobe by hemodilution and a normal diuretic response to hydration. The observations of Porter (26) that adrenal steroids suppress both spontaneous electrical activity and the excitatory effects of epinephrine on the hypothalamus in adrenalectomized monkeys provides some experimental evidence for this theory.

The fact that normal subjects demonstrate similar suppression of neurohypophyseal response to nicotine with steroid therapy, usually associated with enhanced free water diuresis exceeding previously established maxima, suggests that vasopressin secretion may not be inhibited completely by water administration even in normal man. This possibility interjects a cautious note in the interpretation of studies performed during water diuresis based upon the assumption that circulating vasopressin has been completely removed from the experimental protocol.

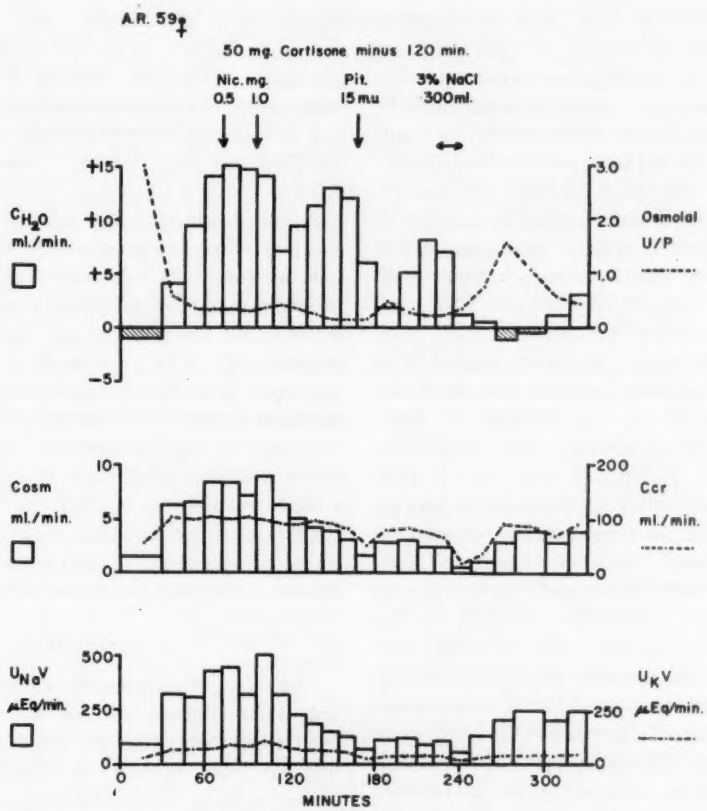

FIG. 12. EFFECT OF CORTISONE ON NEUROHYPOPHYSEAL FUNCTION IN ADDISON'S DISEASE. Sustained hydration of 1.2 L.

The observation of a dual action of fluorocortisol on vasopressin release mechanisms was of particular interest. This steroid is as potent as aldosterone in its effect on mineral metabolism, but also possesses greater glucocorticoid effects than aldosterone (*27*), which could result in a cortisol-like action on neurohypophyseal secretion. The occasional enhancement of the neurohypophyseal

TABLE II

Summary of effects of adrenal streoids in normal and Addisonian subjects *

Group	Steroid	No. of studies	C_{H_2O}			U_{osm}			C_{osm}			Nicotine threshold†		
			Inc.	Decr.	Same	Inc.	Decr.	Same	Inc.	Decr.	Same	Inc.	Decr.	Same
Normal subjects	F	6	6	0	0	1	4	1	3	2	1	5	0	0
	F1-F	4	1	2	1	0	2	2	0	4	0	2	1	1
	DCA	2	1	0	1	0	1	1	1	1	0	1	0	1
Addison's disease	F	5	5	0	0	1	4	0	2	2	1	4	0	0

* F = cortisol-like steroids, F1-F = fluorocortisol, DCA = desoxycorticosterone acetate, inc. = increase, decr. = decrease.

† Nicotine threshold refers to the dose of intravenous nicotine required to produce an unequivocal decrease in C_{H_2O} and rise in U_{osm}.

response to nicotine by Fl-F, however, suggests that potent mineralocorticoids actually may increase the sensitivity of vasopressin release mechanisms in some instances, an action which may be of fundamental importance in the endocrine regulation of the volume and tonicity of the body fluids. The observation that DCA exerted a glucocorticoid-like action on neurohypophyseal function in two normal subjects is difficult to explain within the framework of this hypothesis. Since both subjects showed similar responses to Fl-F, and since DCA is known to produce extremely variable effects on electrolyte and water metabolism in normal subjects, similar variability in the action of mineralocorticoids on neurohypophyseal function in normal man should not be too surprising.

There is ample evidence to show that marked alterations in total solute excretion mask the action of vasopressin on renal tubular reabsorption of water. Although increased sodium and total solute excretion were occasionally observed with administration of cortisol-like steroids in this study, there were sufficient data showing definite suppression of nicotine antidiuresis during periods of constant sodium and total solute excretion, without apparent alteration in the intensity or duration of the antidiuretic response to hypertonic saline or exogenous vasopressin, to corroborate the conclusion that the glucocorticoids suppress nicotine-induced vasopressin release. Further substantiation of this conclusion was apparent in several studies in which marked and sustained sodium retention was produced initially by a potent mineralocorticoid following which glucocorticoid administration clearly suppressed nicotine antidiuresis without altering the depressed rates of sodium and solute excretion or the effects of hypertonic saline and vasopressin.

The hypothesis that glucocorticoids suppress vasopressin release does not deny other possible actions of adrenal steroids on water diuresis, such as that associated with increases in renal hemodynamics (28), but only slight effects of steroids on glomerular filtration rate (C_{cr}) were observed in this study. The acute decreases in C_{cr} frequently apparent in this study during nicotine antidiuresis suggest a possible effect of nicotine on the vascular system and the renal circulation which could explain the increased tubular reabsorption of water without implicating a role of vasopressin release in this phenomenon. However, mean creatinine clearances for the entire periods of nicotine antidiuresis were found to be relatively constant except in one patient with Addison's disease, and this lack of effect of nicotine on glomerular filtration rate has also been observed by others (29).

A conclusion that the adrenal steroids exert a direct effect on permeability of renal tubular cells to water (2) also would have been tenable in interpretation of our data if studies of neurohypophyseal function had not been performed simultaneously with measurements of free water clearance. The definite association between acute increases in free water excretion and marked alterations in neurohypophyseal secretory mechanisms provides circumstantial evidence to favor a causal relationship between the two phenomena. Further clarification of this hypothesis must await actual measurement of vasopressin levels in the plasma during adrenal steroid administration.

SUMMARY

The effect of various adrenal steroids on standard water diuresis and on the antidiuretic response to vasopressin, nicotine and hypertonic saline has been evaluated in normal and in Addisonian subjects. Glucocorticoids regularly suppress the neurohypophyseal response to intravenous nicotine without altering release of vasopressin produced by infusion of hypertonic saline or the antidiuretic effects of exogenous vasopressin. In several instances the salt-retaining hormone, fluorocortisol, has been shown to sensitize the response of the neurohypophysis to nicotine.

The data suggest that glucocorticoids enhance water diuresis in normal and in Addisonian subjects by inhibiting the hypothalamic control of vasopressin release, rather than by competition with vasopressin at the renal tubular level.

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SERUM β-LIPOPROTEIN LEVELS IN HEALTH AND DISEASE, AS DETERMINED BY THE NEPHELOMETRIC METHOD WITH AMYLOPECTIN SULFATE AS REAGENT *

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An elevation of certain serum lipoproteins in atherosclerosis has first been described by Gofman and co-workers (1). Extensive studies of the giant serum lipoprotein molecules and of their role in atherosclerosis have subsequently been carried out by these authors, using the analytical ultracentrifuge as a tool (2–5). Their results showed a statistically significant increase of certain low density lipoprotein fractions in patients and in experimental animals with atherosclerosis. Similar findings have been reported by Barr, Russ and Eder (6), using the technique of fractional protein precipitation.

Both methods of serum β-lipoprotein determination are time consuming, require intricate and costly equipment, and may necessitate specially trained personnel. In the search for simpler and quicker methods, particularly in view of the desirability of screening large human populations, the specific interaction of serum β-lipoprotein with macromolecular polyanions has provided a novel principle for the assay of this serum protein. The study of the lipoprotein-polyanion interaction has shown that these two components are capable of forming several types of complexes of varying solubility and affinity (7, 8), and that the solubility of the complexes is governed by the chemical and physicochemical nature of the polyanion (9). Amylopectin sulfate has been found to produce a highly insoluble complex with human serum β-lipoprotein (10), and nephelometric evaluation of the concentration of this complex has been applied to the quantitative determination of β-lipoprotein (11). Other macromolecular polyanions have been suggested for the same purpose, such as heparin in the presence of calcium (12), dextran sulfate (13, 14), and carrageenin (15).

Recent studies dealing with the influence of the chemical and physicochemical nature of macromolecular polyanions and their interaction with human serum β-lipoprotein (16) have shown that a variation in the molecular weight of the polyanion changes the solubility of the complex and, hence, modifies the results of lipoprotein analysis. For the procedure of determining this protein, only those polysaccharide sulfates appear to be suitable, therefore, which can be easily obtained at a highly reproducible degree of polymerization, without requiring prior hydrolysis or fractionation. The sulfation of amylopectin extracted from cornstarch by the method of Schoch (17) appears to provide a highly reliable reagent which fulfills all these requirements and which, in addition, has been found to have the advantage of yielding more insoluble complexes with human serum β-lipoproteins than practically any other polyanion (16).

The present paper emphasizes the reproducibility of the data for β-lipoprotein obtained with amylopectin sulfate as a reagent, provides clinical findings on a large population of over 2,000 subjects, and demonstrates the practical applicability of this extremely simple and rapid method, in particular for large-scale screening programs.

EXPERIMENTAL

Method. The procedure for lipoprotein determination described previously was followed (11). Aliquots of 0.1 ml of serum were diluted to 25 ml with "Tris" buffer, pH 8.6, ionic strength 0.05. After nephelometric determination of the light scattering intensity of this solution, 0.15 ml of a 0.1 per cent solution of amylopectin sulfate was added, and a second nephelometric reading was made, not less than 10 minutes and not more than 30 minutes after the addition of the reagent. All nephelometric readings were carried out in either a Coleman Model 7 Photo-Nephelometer or in a Coleman Model 9 Nepho-Colorimeter. Commercial nephelos standards with N = 75 to N = 78 served as reference turbidities.[1]

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[1] Manufactured and calibrated by the Coleman Instrument Co., Inc.

The data for β-lipoprotein thus obtained had the denomination of nephelometric indices expressed in nephelos units, and represent the increase in turbidity observed after addition of amylopectin sulfate to serum. These values are numerically identical with those obtained in a Fisher Nefluoro-Photometer, using as reference turbidities freshly prepared and stabilized barium sulfate suspensions, as described earlier (11). Multiplication of the nephelometric index by the empiric factor of 12.1 yields β-lipoprotein values in milligrams per 100 ml of serum (11).

Source of serum samples. Serum from individuals without known disease was received from the following sources: Employee Relations Department of the Medical Division of the E. I. Du Pont de Nemours & Company; Health Center of the International Ladies' Garment Workers' Union, Boston, Mass.; Medical Department of the New England Telephone and Telegraph Co.; Medical Department of the Socony Mobil Oil Company, Inc.; Lions Clubs of Brighton, Dedham, Foxboro, Medford, and Parkway (West Roxbury), Mass.; the Lexington Kiwanis Club; and Boston Chapter of the Alpha Kappa Alpha Sorority.

The following hospitals and institutions have contributed serum samples from patients: Boston City Hospital, New England Center Hospital, Holy Ghost Hospital in Cambridge, (all in Massachusetts), and the Memorial Hospital, New York.

All serum samples were shipped by air mail, or delivered locally by messenger. They were stored in a cool place at 2° to 6° for periods of time not exceeding 3 weeks. Freezing of the samples was avoided.

Characterization of amylopectin sulfate as the barium salt. The potency of each batch of amylopectin sulfate in producing turbidity with an excess of barium ions in acid medium was measured as follows: 0.3 ml of a 1 per cent aqueous solution of the polyanion was added to a mixture of 1 ml of 10 per cent barium acetate with 24 ml 0.2 N HCl, and the increase in turbidity was determined nephelometrically at least 10 minutes but not later than 15 minutes after the addition of the polyanion. This barium turbidity of the polyanion was expressed in nephelos units. *Total serum cholesterol* was determined by the method of Schoenheimer and Sperry (18).

Statistical analysis. Expressions and notations of Dixon and Massey's (19) textbook are used throughout this work. Statistical significance of the difference between the data of two groups has been expressed in t values. At a 1 per cent level of significance, differences are considered to be highly significant when $t > 2.6$ or $t < -2.6$ with more than 120 degrees of freedom. Since the comparison of each group was made with a normal sample of over 900 individuals, the degrees of freedom exceeded 120 in each case.

RESULTS

Testing of amylopectin sulfate for adequacy as a reagent. The quotients of barium turbidity (obtained in acid medium) to sulfur content (per cent S of the potassium salts) for 13 individual batches of corn amylopectin sulfate and for three batches of potato amylopectin sulfate are shown in Table I. These quotients are compared to the potency of each polyanion to produce a turbidity with serum β-lipoprotein at pH 8.6; the latter value is expressed in per cent of the nephelometric index obtained with a reference polyanion (corn amylopectin sulfate, batch 11), and the same source of β-lipoprotein, i.e., the same serum sample. The following conclusions can be drawn from the data in Table I.

1) Most samples of corn amylopectin sulfate exhibit serum turbidity values ranging between 97.5 and 102.5 per cent of that of the reference polyanion. These batches may, thus, be considered to yield reproducible β-lipoprotein values, and to be adequate as a reagent.

2) All batches of amylopectin sulfate which exhibit serum turbidity values between 97.5 and 102.5 per cent of that of the reference polyanion show quotients of barium turbidity to sulfur content (bt/S quotients) ranging from 4.95 to 5.4, while samples of amylopectin sulfate with lower serum turbidity values exhibit markedly lower bt/S quotients. The determination of the bt/S quotient of an amylopectin sulfate preparation appears to provide, therefore, a valuable criterion for the adequacy of the polyanion as a reagent for β-lipoprotein, wherever a reference preparation of amylopectin sulfate is not available.

3) While amylopectin sulfate preparations with low sulfur contents (10 per cent or less), obtained on purpose by incomplete sulfation (16), give low serum turbidity values, maximum turbidity can be reached with moderately sulfated corn amylopectin (13 per cent or more). No explanation is available, however, for the existence of an occasional batch of amylopectin sulfate with more than 13 per cent sulfur, but with a low serum turbidity (batch 15). The low bt/S quotient of such a batch easily allows its recognition.

4) The potency of corn amylopectin sulfate in producing a turbidity with serum β-lipoprotein is the same, irrespective of the crop of cornstarch used to obtain the amylopectin, and irrespective of the batch of amylopectin extracted from the cornstarch.

TABLE I

Comparison of the serum turbidity of different batches of amylopectin sulfate by the use of the solubility of their barium salts

Source of amylopectin sulfate	Batch no.	S	Quotient of barium turbidity* to per cent sulfur	Turbidity with serum at pH 8.6†
		%		
Cornstarch, crop A	11‡	16.67	5.1	100
Cornstarch, crop A	12	15.8	4.95	99.5
Cornstarch, crop A	18	15.6	5.35	98.5
Cornstarch, crop B	19	13.6	5.0	97.5
Cornstarch, crop B	26	13.2	5.0	101.5
Cornstarch, crop B	27	15.9	5.4	102.5
Cornstarch, crop B	30	14.3	5.25	101
Cornstarch, crop B	32	13.8	5.2	98.5
Cornstarch, crop B	33	13.75	5.1	102
Cornstarch, crop B	15	14.3	4.55	80
Cornstarch, crop B	16	10.4§	0.25	79
Cornstarch, crop B	21	8.0§	0.3	56
Potato starch‖	20	14.0	4.25	82
Potato starch‖	22	17.5	3.55	94.5
Potato starch‖	25	11.5	2.8	72

* Definition of barium turbidity in acid medium, see text.
† Expressed in per cent of the nephelometric index observed with a reference polyanion (corn amylopectin sulfate, batch 11), using the same serum sample.
‡ Reference polyanion, serum turbidity set at 100.
§ Low sulfur content, obtained on purpose by incomplete sulfation.
‖ Potato amylopectin (Ramalin) obtained from Stein, Hall & Co., N. Y.

5) Sulfated potato amylopectin yields generally lower serum turbidities than does corn amylopectin sulfate with corresponding sulfate content.

Normal population. The age distribution and the proportion of males and females in the normal population sample used in the present investigation, as well as mean values for β-lipoprotein in the various age groups, are seen in Table II.

It is evident from the data in Table II that there is almost no difference between the mean β-lipoprotein values in normal males and females. Although the number of women with no known dis-

TABLE II

β-Lipoprotein in the normal population used in this study

Age groups	No.	β-Lipoprotein* (found)		Mean values of β-lipoprotein calculated from regression line†
		mg %		mg %
15–19	1	441		
20–24	1	502		
25–29	24	657	[117]	635
30–34	43	626	[138]	645
35–39	34	661	[108]	655
40–44	236	664	[151]	665
45–49	243	673	[169]	675
50–54	181	715	[170]	685
55–59	113	686	[149]	695
60–64	60	675	[197]	705
65–69	5	767	[180]	715
70 and over	1	641		
All males	893	678	[159]	
All females	49	681	[164]	
Total	942	678	[158]	

* Mean values; standard deviation in brackets.
† See text.

TABLE III

β-Lipoprotein values in cardiovascular disease

Diagnosis	Sample no.	β-Lipoprotein*	*t* Value†
		mg %	
Normals (see Table II)	942	678 [158]	
Myocardial infarcts (total)	220	780 [172]	8.5
Anterior	57	790 [162]	
Anterior on anticoagulants‡	14	709 [133]	
Posterior	38	772 [161]	
Posterior on anticoagulants‡	11	783 [158]	
Unclassified	99	785 [188]	
Unclassified on anticoagulants‡	1	944	
Multiple myocardial infarcts	11	841 [225]	3.4
Coronary artery disease (total)	76	824 [166]	7.7
Without anticoagulants	72	822 [167]	
On anticoagulants‡	4	872 [154]	
Arteriosclerotic heart disease (total)	36	808 [241]	4.8
Without anticoagulants	29	826 [256]	
On anticoagulants‡	7	730 [151]	
Miscellaneous cardiovascular diseases (total)	15	668 [150]	−0.24
Hypertension	6	678 [120]	
Cardiac disease, unclassified§	9	662 [174]	
Total cardiovascular diseases	358	790 [182]	11.0

* Mean values, standard deviations in brackets.

† The *t* value expresses the statistical significance of the difference in β-lipoprotein levels between each individual group and the normals. A *t* value of over 2.6 (or of less than −2.6) indicates that the difference is highly significant ($p < 0.01$).

‡ Danilone, 2-phenyl-1,3-indanedione, Schieffelin & Co.

§ Includes 1 Adams-Stokes syndrome with complete heart block, 1 enlarged heart, and 7 cases of unspecified heart disease.

ease was small in comparison to the corresponding male population, the *t* value of statistical significance for these two groups of normals is as low as $t = -0.21$. This indicates that the whole population sample is well suited for a normal control group in this study, in spite of the relatively small number of females it contains.

While the data at first sight seem to show that in the age groups above 25 years β-lipoprotein does not depend on age, closer observation indicates that β-lipoprotein in a few of the 5-year age groups would significantly differ from the mean of the whole normal population, e.g., the 50 to 54 year age group exhibits a *t* of 2.85, and the 30 to 34 year age group a *t* of −2.1. This appears unlikely, however. On the other hand, when the relationship of age to β-lipoprotein levels is expressed by a regression line calculated from the mean values of Table II, this regression line, with the parameter b = 2.00, shows that the theoretical mean values for β-lipoprotein gradually and slowly increase with age, and it leads to calculated values for each 5-year age group shown in the last column of Table II. These latter values agree well with the mean values found experimentally.

The differences in mean β-lipoprotein values between the normal control group and groups of patients with certain diseases were considerable (*t* greater than 3.7 or less than −3.7), as shown in the following four tables. It was not felt necessary, therefore, to take the age relationship into account, in particular since the normal population ranged in age from 25 to 70 years, with 89 per cent of the subjects being over 40 years of age. The normal control group, exhibiting a mean age of 45.3 years, thus resembled closely the various patient populations with respect to age distribution. Consequently, all *t* values given in Tables III through VI were calculated on the basis of a mean β-lipoprotein value for the whole normal group, i.e., 678 mg per 100 ml.

TABLE IV

β-Lipoprotein in cerebrovascular disease

Diagnosis	Sample no.	β-Lipoprotein*	*t* Value†
		mg %	
Normals (see Table II)	942	678 [158]	
Cerebral thrombosis (total)	132	764 [203]	5.6
Without hypertension	97	737 [203]	
With hypertension	35	837 [188]	
Cerebral hemorrhage (total)	12	851 [250]	3.7
Without hypertension	7	724 [212]	
With hypertension	5	1,030 [190]	
Cerebrovascular accidents, unclassified (total)	119	799 [250]	7.3
Without hypertension	109	788 [234]	
With hypertension	10	916 [384]	
Multiple cerebral thromboses (total)	37	825 [188]	5.5
2 Strokes	8	871 [178]	
2 Strokes with hypertension	2	876	
3 Strokes	3	770 [87]	
3 Strokes with hypertension	1	759	
More than 3 strokes	16	830 [241]	
More than 3 strokes with hypertension	7	782 [53]	
Miscellaneous cerebrovascular diseases (total)	7	693 [145]	0.26
Without hypertension‡	5	697 [166]	
With hypertension	2	687	
Total cerebrovascular diseases	307	787 [223]	9.4

* Mean values, standard deviations in brackets.

† The *t* value expresses the statistical significance of the difference of β-lipoprotein levels between each individual group and the normals. A *t* value of over 2.6 or less than −2.6 indicates that the difference is highly significant ($p < 0.01$).

‡ Includes 2 cases of generalized cerebral arteriosclerosis, 2 of cerebral vascular insufficiency, and 1 organic brain syndrome with severe arteriosclerosis.

TABLE V

β-Lipoprotein in arteriosclerosis

Diagnosis	Sample no.	β-Lipoprotein*	*t* Value†
		mg %	
Normals (see Table II)	942	678 [158]	
Cardiovascular disease (see Table III)	358	790 [182]	11.0
Cerebrovascular disease (see Table IV)	307	787 [223]	9.4
Simultaneous cardiovascular and cerebrovascular disease (total)	63	803 [194]	6.0
Unclassified	15	782 [186]	
With hypertension	21	823 [218]	
With coronary insufficiency	12	785 [168]	
With myocardial infarcts	15	813 [202]	
Other arteriosclerotic syndromes (total)	35	753 [157]	2.8
Carotid artery thrombosis	11	830 [154]	
Basilar artery insufficiency	18	691 [143]	
Miscellaneous‡	6	800 [149]	
Total of arteriosclerosis	763	788 [199]	12.75

* Mean values, standard deviations in brackets.

† The *t* value expresses the statistical significance of the difference of β-lipoprotein levels between each individual group and the normals. A *t* value of over 2.6 or of less than −2.6 indicates that the difference is highly significant ($p < 0.01$).

‡ Includes 1 Alzheimer's disease, 1 case of senility and 4 cases of general arteriosclerosis.

β-Lipoprotein in arteriosclerosis. The data in Tables III to V show that the nephelometric procedure for β-lipoprotein is well qualified to demonstrate the increase in concentration of this serum protein in cardiovascular and cerebrovascular diseases, as has already been indicated in a preliminary communication (20). The elevated *t* values are an expression of the high significance of these increases. In a total of 763 patients with various syndromes due to arteriosclerosis, the mean β-lipoprotein was elevated from the normal level of 678 to 788 mg per 100 ml with a *t* value of 12.75. The high standard deviations of the means of 158 and 199, respectively, characterize the large spread of the individual data. This indicates a considerable amount of overlapping between the values of normal subjects and those of patients.

It can also be seen from the data in Tables III

TABLE VI

β-Lipoprotein in diseases and conditions other than arteriosclerosis *

Disease	Sample no.	β-Lipoprotein†	*t* Value‡
		mg %	
Normals (see Table II)	942	678 [158]	
Kidney diseases (total)	51	785 [208]	4.7
Unclassified	12	685 [173]	
Chronic pyelonephritis	26	790 [231]	
Acute and chronic pyelonephritis	3	857 [223]	
Acute glomerulonephritis	2	909 [279]	
Chronic glomerulonephritis	6	828 [99]	
With malignant hypertension	1	1,118	
Gouty nephritis	1	788	
Liver diseases (total)§	21	808 [237]	3.7
Malignant diseases (total)	91	709 [178]	1.8
Solid tumors‖	64	711 [173]	
Lymphomas¶	15	750 [203]	
Leukemias**	12	644 [163]	
Hemorrhagic diseases††	3	832 [68]	1.7
Arthritis	9	650 [235]	−0.52
Diabetes	81	764 [201]	4.6
Neurological diseases (total)	13	575 [113]	−2.3
Multiple sclerosis	4	626 [119]	
Parkinson's disease	7	567 [111]	
Miscellaneous‡‡	2	499	
Essential hypercholesterolemia	29	974 [262]	9.7
Obesity	9	764 [201]	1.85
Other diseases§§	14	686 [157]	0.20

* Clinical findings on the patients whose lipoprotein data were compiled in this table did not indicate the presence of cerebrovascular or cardiovascular disease.

† Mean values, standard deviations in brackets.

‡ The *t* value expresses the statistical significance of the difference of β-lipoprotein levels between each individual group and the normals. A *t* value of over 2.6 or of less than −2.6 indicates that the difference is highly significant ($p < 0.01$).

§ Includes 9 cases of cirrhosis, 2 of infectious hepatitis, 7 cases of hepatitis (unclassified), and 3 cases of unspecified liver disease.

‖ Fifteen carcinoma of the breast, 14 of the prostate, 8 of the lung, 5 of the colon, 5 of the stomach, 4 of the esophagus, 3 of the rectum, 3 of the cervix, 1 each of the tongue, throat, bladder and uterus, 1 melanoma, 1 hypernephroma, and 2 unspecified carcinomas.

¶ Seven cases with Hodgkin's disease, 4 lymphomas, 2 lymphosarcomas, and 2 multiple myelomas.

** Ten myelogenous and 2 lymphatic leukemias.

†† Three cases with anemia.

‡‡ One case each with epilepsy and cerebral palsy.

§§ Three cases with psychoneurosis, 2 with lues, and 1 with each of the following conditions: infectious mononucleosis, tuberculosis, prostatic hypertrophy, aortic aneurysm, gall stones, retinitis, Pott's disease, Boeck's sarcoid, and fractured hip in an 84-year-old woman.

TABLE VII

Correlation between total serum cholesterol and nephelometric β-lipoprotein

	High or low cholesterol*	No.	Mean $\bar{X}$	Standard deviation s	Quotient $s/\bar{X}$	Correlation coefficient
Total cholesterol (*mg %*)	A	82	224	60.8	0.271	
	B	13	361	92.1	0.255	
β-Lipoprotein (*mg %*)	A	82	667	143	0.214	
	B	13	923	207	0.224	
Ratio $\frac{\text{total cholesterol}\dagger}{\beta\text{-lipoprotein}}$	A	82	0.341	0.081	0.237	0.61
	B	13	0.395	0.0575	0.146	0.79

* A = total cholesterol not exceeding 300 mg %; B = total cholesterol over 300 mg %.
† This ratio has been calculated for the two measurements of each individual serum sample.

to V that the treatment of cardiovascular patients with such anticoagulants as Danilone,[2] does not significantly reduce their serum β-lipoprotein. β-Lipoprotein levels were not found to be elevated in patients with uncomplicated hypertension, but in patients with hypertension associated with cerebrovascular disorders, β-lipoprotein was frequently higher than in patients with cerebrovascular disease alone.

β-Lipoprotein in other diseases. Significant increases in β-lipoprotein were shown not to be specific for patients with arteriosclerosis alone, but to occur also in a small number of totally unrelated conditions, namely, kidney diseases, liver diseases, and diabetes, as can be seen from the data in Table VI. On the other hand, patients with malignant diseases did not exhibit significant β-lipoprotein changes. Normal levels were also found in small numbers of patients with arthritis and with hemorrhagic, neurological and miscellaneous other diseases. The mean value of β-lipoprotein in obese persons appeared to be increased; this increase is not significant, however, due to the small number of subjects studied and to the high spread of the values, characterized by the high standard deviation.

Correlation between β-lipoprotein and total cholesterol. Elevated β-lipoprotein values in hypercholesterolemia, as seen in Table VI, were to be expected. At serum cholesterol values below 300 mg per 100 ml, however, there appears to be little relationship between the nephelometrically determined β-lipoprotein and the total cholesterol values. Table VII shows results obtained with a population sample of 95 individuals chosen at random. Among these, 82 subjects had total cholesterol values not exceeding 300 mg per 100 ml. This group contained 40 normal individuals, 17 patients with arteriosclerosis, 7 with neurological disorders, 6 with diabetes, 5 obese persons, 3 cases with psychoneurosis, 1 cancer patient, 1 arthritic, 1 individual with a fractured hip, and 1 patient with Pott's disease. It is evident from the data in Table VII that the quotient of standard deviation over mean value ($s/\bar{X}$, see last column) is rather similar for the total cholesterol and for the β-lipoprotein values in this group, denoting an expected similarity of the sampling distributions of these two characteristics. If the β-lipoprotein concentrations were related to those of cholesterol, the ratios of these two measurements for each individual subject would tend to be constant, i.e., the quotient of standard deviation of the mean of these individual ratios over the mean of the ratios (sixth column) would be much smaller than the corresponding $s/\bar{X}$ ratios for either total cholesterol or β-lipoprotein. The data in Table VII show that this is not the case, however. The independence of β-lipoprotein from total cholesterol is further illustrated by a low correlation coefficient of $R = 0.61$, as well as by a very low rank correlation coefficient of $R_s = 0.34$, calculated from the experimental data. Measurements of total cholesterol and of β-lipoprotein would be interchangeable only if the correlation coefficients were 1, or very close to unity. In the second group consisting of 13 individuals with hypercholesterolemia, the $s/\bar{X}$ ratio is considerably lower

[2] Branch of 2-phenyl-1,3-indanedione, manufactured by Schieffelin & Co.

than the corresponding ratios for both cholesterol and lipoprotein, while the correlation coefficient is significantly higher, denoting a higher degree of correlation between the two factors at high total cholesterol levels.

DISCUSSION

In evaluating the determination of serum β-lipoprotein obtained by the nephelometric method, one has to bear in mind that, on account of the marked heterogeneity of the so-called low density or β-lipoproteins, this procedure cannot be expected to exhibit a specificity for exactly the same spectrum of lipoproteins as other techniques which are based on entirely different properties of the lipoprotein, such as ultracentrifugal flotation or paper electrophoresis. While it has been demonstrated (11) that the present method measures only those lipoproteins which migrate as β-globulins upon electrophoresis of whole serum at pH 8.6, and that all of the β-lipoproteins are involved in the precipitation with amylopectin sulfate at pH 8.6, an exact correlation between ultracentrifugal flotation rates of lipoproteins (S_f-values) and their precipitation by amylopectin sulfate is rather difficult to establish Nevertheless, it has been found that the flotation rate of β-lipoprotein fractions obtained at small amylopectin sulfate concentrations is lower than that of fractions precipitated at higher polyanion concentrations (21). It appears likely that β-lipoproteins with flotation rates between S_f 2 and 12 with a peak at S_f 4 have the highest affinity for amylopectin sulfate, and that the affinity slowly diminishes as the flotation rate increases, while chylomicrons are not precipitated at all.

The absence of a correlation between β-lipoprotein results obtained by the nephelometric method and data on lipoproteins of any of the routinely computed S_f-groups made it necessary to establish the clinical relevance of the new method in an independent manner. The data presented here show a highly significant statistical difference between the nephelometric β-lipoprotein values of normal individuals ranging from 25 to over 70 years of age and those of patients with cardiovascular disease, with cerebrovascular disease, with other arteriosclerotic syndromes, with diabetes, kidney and liver diseases, and, of course, with hypercholesterolemia.

It is evident, therefore, that the nephelometric method, because of its simplicity, rapidity and reproducibility, has proven its value as a clinical tool for the determination of serum β-lipoprotein. Although this method is not intended to replace the more intricate, costly and time consuming ultracentrifugal and electrophoretic techniques, the nephelometric method is considered to be of particular usefulness as an independent measurement of serum β-lipoprotein, especially for the purpose of screening great numbers of individuals, such as in large scale nutritional studies and in life insurance surveys of large populations, as well as for experimental work with small animals where the available amounts of serum are limited.

The comparison of nephelometric β-lipoprotein values with total cholesterol data showed that a certain correlation between these two factors exists. This was to be expected, since the latter is a component part of the molecule of the former. Nevertheless, it is evident that the correlation between these two serum constituents is so small that the determination of one in a given population sample will not lead to the same information as does the measurement of the other. The correlation becomes closer, however, as the cholesterol levels increase.

The use of corn amylopectin as a reagent for β-lipoprotein appears appropriate because its molecular weight is more controllable than that of any other macromolecular polysulfate esters which have been suggested as alternate reagents (12–15). The elimination of the molecular weight of the polyanion as a variable thus assures a good reproducibility between the results of different laboratories.

In view of the extensive literature on elevated β-lipoprotein in arteriosclerosis and in certain other conditions (1–6), no comments on the present findings appear necessary.

However, in contrast to the present data, serum lipoprotein or serum lipid levels in patients with neoplastic diseases have been reported to be increased by some workers (22), and decreased by others (23). More work in this direction, in particular, on a larger patient population and with clear distinction between the different types of malignant diseases, will be necessary to draw final conclusions on the relationship of β-lipoprotein to neoplastic growth.

SUMMARY

1. Serum β-lipoprotein was determined in over 2,000 individuals by the nephelometric method with amylopectin sulfate as reagent.

2. Since the molecular weight of polyanionic reagents for β-lipoprotein is known to influence the reaction markedly, the reproducibility of the results was ascertained by choosing corn amylopectin sulfate, the molecular weight of which is easily controllable, as the reagent.

3. A method for the standardization of the reagent was devised, based on the determination of its sulfur content and of the turbidity formed by the addition of barium ions to amylopectin sulfate in an acid medium. The results with different batches of the reagent were easily reproducible. The origin of the amylopectin from different crops of cornstarch, the extraction of the amylopectin from the starch, and the sulfation of amylopectin have no influence on the quality of the reagent.

4. A normal population of 942 individuals ranging from 25 to 70 years of age, with 89 per cent being over 40 years and with an average age of 45.3 years, exhibited a mean β-lipoprotein value of 678 mg per 100 ml, with a standard deviation of 158. Mean β-lipoprotein values increased with age from 635 mg per 100 ml for the 25 to 29 year age group, to 715 mg per 100 ml for the 64 to 69 year age group.

5. In 763 individuals with arteriosclerosis, including 358 with cardiovascular disease, 307 with cerebrovascular disease, and 63 with the simultaneous occurrence of these two conditions, the mean lipoprotein level was elevated to 788 mg per 100 ml. This increase is highly significant ($t = 12.75$). Treatment of the cardiovascular patients with the anticoagulant, Danilone, does not significantly reduce their serum β-lipoprotein. Patients with hypertensive cerebrovascular disorders usually have higher β-lipoprotein levels than those with cerebrovascular disease without hypertension, while hypertension when occurring alone does not appear to be accompanied by changes of serum β-lipoprotein.

6. Increases in the concentrations of serum β-lipoprotein have also been observed in kidney diseases (mean = 785 mg per 100 ml in 51 cases, $t = 4.7$), in liver diseases (mean = 808 mg per 100 ml in 21 cases, $t = 3.7$), in diabetes (mean = 764 mg per 100 ml in 81 cases, $t = 4.6$) and in hypercholesterolemia (mean = 974 mg per 100 ml in 29 cases, $t = 9.7$). No significant changes in serum β-lipoprotein were observed in malignant diseases (mean = 709 mg per 100 ml in 91 cases, $t = 1.8$), or in limited numbers of patients with anemia, arthritis, neurological diseases, or obesity.

7. The correlation between total serum cholesterol and nephelometric β-lipoprotein is low; it is somewhat higher in patients with hypercholesterolemia.

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STUDIES ON STARLING'S LAW OF THE HEART. III. OBSERVATIONS IN PATIENTS WITH MITRAL STENOSIS AND ATRIAL FIBRILLATION ON THE RELATIONSHIPS BETWEEN LEFT VENTRICULAR END-DIASTOLIC SEGMENT LENGTH, FILLING PRESSURE, AND THE CHARACTERISTICS OF VENTRICULAR CONTRACTION *

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Although 45 years have now elapsed since Starling's Linacre lecture on the "Law of the Heart Beat" (1), considerable controversy is still centered around the question of the applicability of this fundamental principle to the human heart. In several investigations the Starling concept has been examined by attempts to reproduce in man those experiments on the Starling heart-lung preparation in which its responses to acute changes in venous return were studied (2–6). In most of these investigations no consistent relationship between the filling pressure on the right side of the heart and the cardiac output was apparent. In experiments on the intact dog (7), Rushmer, Smith and Franklin have shown that the augmentation of cardiac work which accompanies muscular exercise is not associated with an increase in left ventricular dimensions. These experiments, when taken together with the observations on man (2–6), have cast considerable doubt on the hypothesis that the Frank-Starling mechanism is involved in the regulation of cardiac performance.

In the present investigation, portions of which have been presented in preliminary form elsewhere (8), attempts were made to examine, in a critical manner, the applicability of Starling's law of the heart to man. To accomplish this, it appeared necessary to relate the end-diastolic fiber length and the end-diastolic filling pressure to the characteristics of ventricular contraction under conditions in which substantial changes in ventricular filling take place. It also seemed desirable to make such observations with the heart functioning with a relatively constant level of circulating catecholamines and of sympathetic stimulation, in view of the importance of these factors on myocardial performance (9).

When the inflow of blood into the left ventricle is not impeded, most of the filling of this chamber occurs during the first portion of diastole, i.e., during the so-called rapid filling phase (10). When the heart rate is slow, this phase of the cardiac cycle is not encroached upon, diastasis is present (11, 12), and changes in the duration of diastole do not profoundly modify ventricular filling. In patients with mitral stenosis, the obstruction to left atrial emptying prevents the left ventricle from filling rapidly during early diastole (12). Accordingly, it might be anticipated that in patients with mitral stenosis, and irregular ventricular rates due to atrial fibrillation, at any given left atrial pressure, beat-to-beat variations in the filling of the left ventricle take place due to alterations in the duration of the filling period. It was considered that if Starling's law of the heart operated in patients with mitral stenosis, the characteristics of each left ventricular contraction should appear to be a function of the previous end-diastolic fiber length or end-diastolic pressure, i.e., beats evidencing a relatively great force of ventricular contraction should be preceded by greater end-diastolic fiber lengths and filling pressures than beats evidencing a relatively smaller force of ventricular contraction. On the other hand, if Starling's law did not operate in these patients, then no clear relationship should be evident between ventricular end-diastolic fiber length or end-diastolic pressure and the characteristics of the subsequent contraction.

* Presented in part before the Fifty-second Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 2, 1960.

METHOD

A total of 30 adult patients with atrial fibrillation who had rheumatic mitral stenosis of sufficient severity to require mitral valvulotomy were studied. In 26 patients observations were made in the course of the operation, which was carried out under nitrous oxide-oxygen anesthesia after induction with thiopental. In 13 patients the length of a segment of left ventricular muscle was continuously recorded by means of the mercury-filled resistance gauge described by Whitney (13) (Figure 1); this gauge has been extensively employed by Rushmer for the measurement of ventricular dimensions in the dog (14), and its physical properties have been described in detail by Lawton and Collins (15). After the chest had been opened, and prior to the valvulotomy, the gauge was sewn to the surface of the left ventricle between its apex and base in such a manner that the sutures avoided the coronary arteries and their main branches (Figure 1). After these recordings had been obtained the gauges were removed from the heart and calibrated by determining the deflection which resulted from known changes in the length of the gauge. In several instances the absolute length of the mercury column could not be measured and only the effects of changes in the length of the gauge were determined.

Alterations in the length of the segment of myocardium to which the two ends of the gauge were sewn modified the length of the rubber tubing and therefore of the column of mercury, thereby changing the electrical resistance offered by the gauge. Thus, the gauge could be utilized to record continuously the length of the segment of myocardium to which its two ends were sewn. Although it is now well established that the contours of the recordings of various dimensions in any given ventricle obtained simultaneously are not identical (16–18), any directional change in one end-diastolic dimension is generally accompanied by a similar directional change in other dimensions of the same ventricle (16, 17).

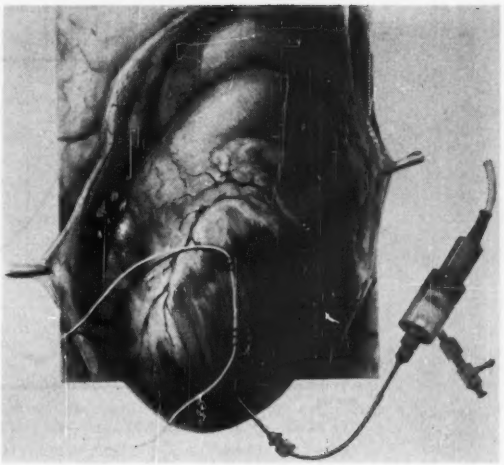

FIG. 1. DRAWING OF THE HEART AS VIEWED IN THE COURSE OF A LEFT THORACOTOMY. The mercury resistance gauge has been sewn to the surface of the left ventricle and left ventricular pressure is measured by means of direct ventricular puncture.

Left ventricular pressures were recorded simultaneously with the segment length in 13 patients (Figure 2) and were obtained without the segment length in an additional 13 patients. The left ventricular pressure was measured by direct puncture with a no. 20 gauge needle which was attached either directly or through a wide-bore nylon connecting tube to a Statham P23D pressure transducer (Figure 1); the baseline for these pressure measurements was the level of the left ventricle and, since the

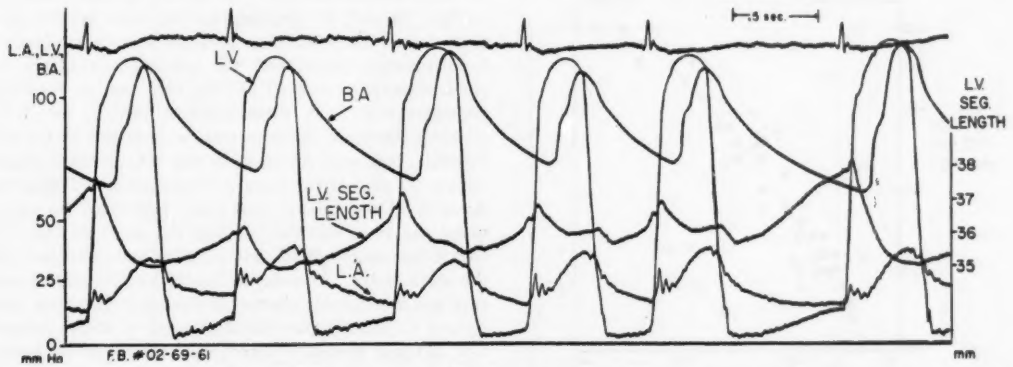

FIG. 2. TYPICAL SIMULTANEOUS RECORDING OF THE ELECTROCARDIOGRAM, LEFT VENTRICULAR (LV), BRACHIAL ARTERIAL (BA) AND LEFT ATRIAL (LA) PRESSURE PULSES AS WELL AS OF LEFT VENTRICULAR SEGMENT LENGTH (LV SEG. LENGTH). It is evident from inspection that the last beat of this tracing is characterized by a greater end-diastolic pressure and segment length than are the preceding beats, and that the peak systolic ventricular pressure, the brachial artery pulse pressure, the duration of systole, and the area beneath the systolic pressure curve are all also greater than in the other beats.

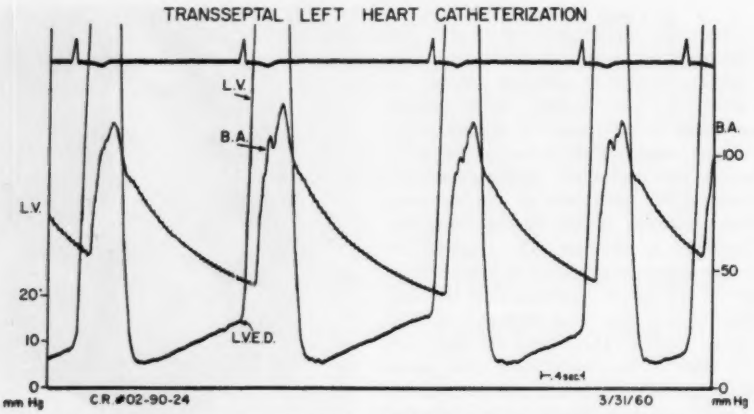

FIG. 3. SIMULTANEOUS LEFT VENTRICULAR (LV) AND BRACHIAL ARTERIAL (BA) PRESSURE PULSES IN A PATIENT WITH PREDOMINANT MITRAL STENOSIS AND ASSOCIATED AORTIC REGURGITATION. These pressure tracings were obtained at left heart catheterization during a time interval when the breath was held. The LV pressure tracing has been amplified in order to increase the accuracy of the measurement of left ventricular end-diastolic pressure (LVED).

pleural cavity and pericardium were open, the recorded pressure is taken as the "effective" left ventricular pressure.

Observations were also carried out on four patients with mitral stenosis and atrial fibrillation who were unanesthetized and intact (Figure 3). Left ventricular pressure was measured through a polyethylene catheter (PE no. 50) at the time of transseptal left heart catheterization (19). In order to avoid the interfering effects of respiration on the "effective" left ventricular pressure, observations were made only on a series of beats which were recorded while the breath was held. The baseline

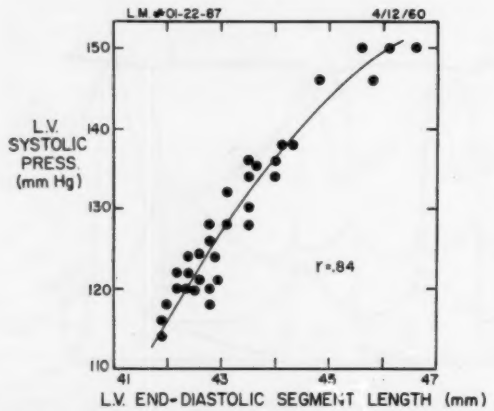

FIG. 4. GRAPH DEPICTING A REPRESENTATIVE RELATIONSHIP BETWEEN THE LEFT VENTRICULAR (LV) END-DIASTOLIC SEGMENT LENGTH AND THE SYSTOLIC PRESSURE OF THE SUBSEQUENT BEAT. Each point represents a single beat.

for these pressures was 5 cm below the sternal angle and although the pressure recorded in this fashion undoubtedly differs slightly from the true "effective" or transmural left ventricular pressure, any *changes* in the latter are reflected accurately in the recorded pressure.

In all 30 patients, systemic artery pressure was recorded from the brachial artery through an indwelling needle attached to a Statham P23D transducer through a nylon connecting tube. All recordings were made on a multi-channel photographic recorder.

Each ventricular contraction was assessed by making four measurements. First, the peak systolic ventricular pressure, a measurement utilized to provide an index of the peak tension developed by the left ventricle, was recorded. Second, the area beneath the entire systolic portion of each left ventricular pressure pulse was obtained by planimetric integration; this permitted calculation of the tension-time index (TTI) for each beat as described elsewhere (20). In these previous studies, the TTI, which is related to the total tension developed by the left ventricle, had been shown to be the hemodynamic determinant of myocardial oxygen consumption. Third, the duration of the tension state was determined by measuring the time interval between the onset of the isometric contraction of the left ventricle and the closure of the aortic valve. Fourth, the brachial artery pulse pressure was determined; changes in this parameter were considered to indicate directional changes in stroke volume (21, 22) and, therefore, directional changes in the extent of shortening of the myocardial fibers.

RESULTS

Analysis of a series of consecutive beats revealed that in any given patient a significant cor-

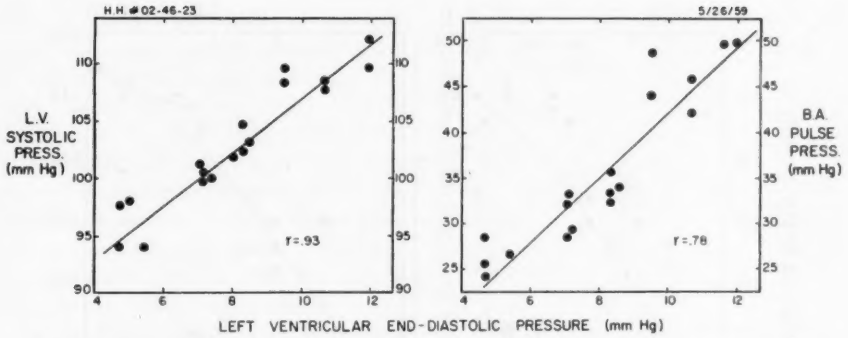

FIG. 5. A REPRESENTATIVE RELATIONSHIP BETWEEN LEFT VENTRICULAR END-DIASTOLIC PRESSURE AND THE SYSTOLIC PRESSURE (LEFT PANEL) AND ARTERIAL PULSE PRESSURE (RIGHT PANEL) OF THE SUBSEQUENT BEAT. Data obtained at the time of operation. Each point represents a single beat and the same series of beats are plotted on both panels.

relation was evident between both the left ventricular end-diastolic segment length (LVEDSL) and left ventricular end-diastolic pressure (LVEDP) and the peak pressure developed by the left ventricle during its next contraction (Figures 4 and 5). Similar correlations between LVEDSL and LVEDP and the TTI (Figure 6), the duration of systole (Figure 7), and the systemic arterial pulse pressure (Figures 5 and 8) of the following beat were also evident. The correlations between the four parameters utilized to characterize ventricular contraction and the LVEDSL, which are illustrated in Figures 4, 6, 7 and 8, are similar to those noted in all 13 patients so studied. The correlations illustrated in Figures 5, 6 and 7 are typical of those observed between LVEDP and the characteristics of ventricular contraction in all but one (Figure 9) of the 26 patients studied at operation. The close correlations between LVEDP and the characteristics of the subsequent contraction, which were evident in the tracings obtained from patients studied at operation, were also present in all four patients studied at the time of transseptal left heart catheterization (Figures 10 and 11).

Since all four parameters of the pressure pulses which were utilized to assess the strength of left ventricular contraction correlated well with both

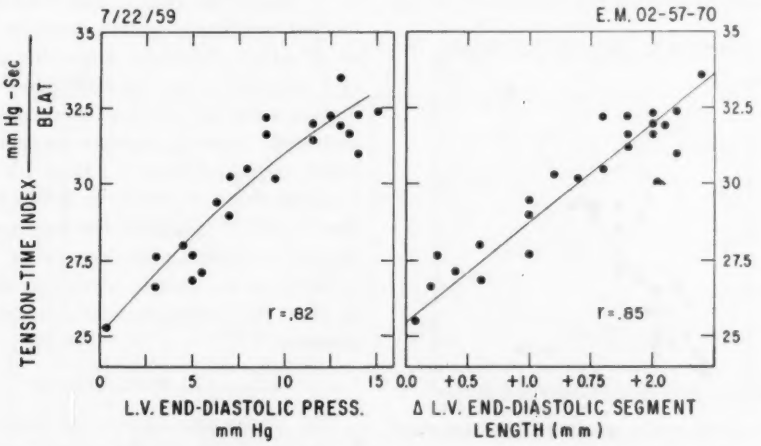

FIG. 6. A REPRESENTATIVE RELATIONSHIP BETWEEN THE TENSION-TIME INDEX AND THE LV END-DIASTOLIC PRESSURE (LEFT PANEL) AND CHANGES IN THE LV END-DIASTOLIC SEGMENT LENGTH (RIGHT PANEL).

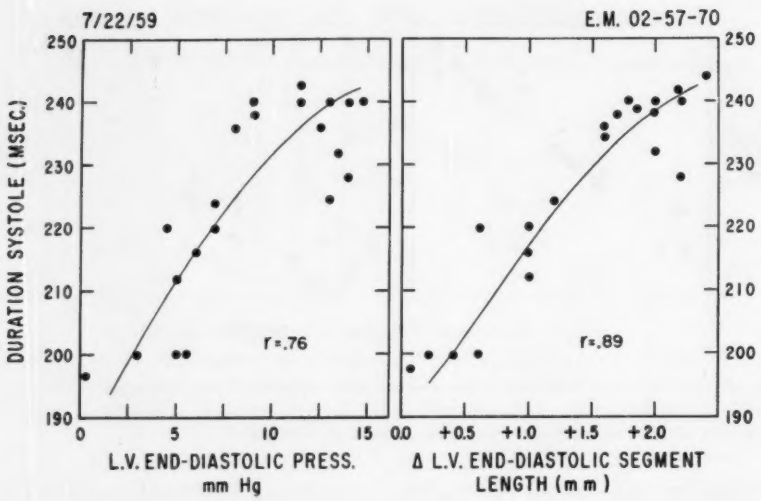

FIG. 7. A REPRESENTATIVE RELATIONSHIP BETWEEN THE DURATION OF SYSTOLE AND LV END-DIASTOLIC PRESSURE (LEFT PANEL) AND CHANGES IN LV END-DIASTOLIC SEGMENT LENGTH (RIGHT PANEL).

the preceding LVEDSL and the LVEDP, it was anticipated that the LVEDSL and LVEDP would, in turn, correlate well with each other. This indeed was the case (Figure 12) in all but one patient (M.L.), in whom unusually large variations in the duration of the ventricular filling period occurred, with some beats following a very brief ventricular filling phase. In this patient it was observed that at any given level of EDP the EDSL was shorter when the duration of filling was less than 0.23 second than when the duration of filling exceeded 0.23 second (Figure 9). In five other patients occasional beats followed filling periods less than 0.20 second; in these beats the EDSL which was observed was shorter than would be anticipated, for the EDP which obtained.

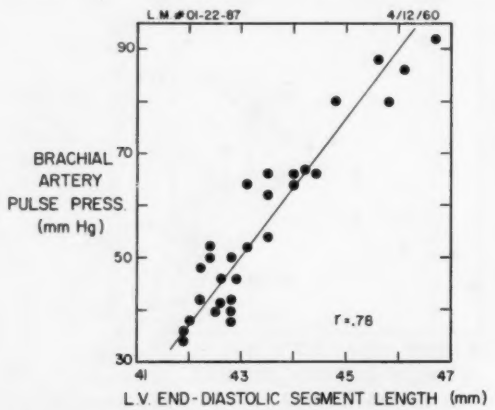

FIG. 8. TYPICAL RELATIONSHIP BETWEEN LV END-DIASTOLIC SEGMENT LENGTH AND THE PULSE PRESSURE OF THE SUBSEQUENT BEAT.

The inconstant relationship between EDSL and EDP in Patient M.L. made it possible to determine which of these two parameters was more closely related to the characteristics of the subsequent contraction. On the left panel of Figure 13 is shown the relationship between LVEDSL and left ventricular systolic pressure in four beats, all of which originated from the same LVEDP (7.5 mm Hg). On the right panel of Figure 13, is shown the relationship between EDP and left ventricular systolic pressure in four beats, all of which originated from the same LVEDSL. It is apparent that alterations in LVEDSL at a constant LVEDP modified the subsequent left ventricular systolic pressure, while changes in LVEDP at a constant LVEDSL did not appear to alter the subsequent left ventricular systolic pressure.

DISCUSSION

The mechanism responsible for the variations in the amplitude of the systolic pressure which occur in atrial fibrillation has been a subject of interest since the description of this arrhythmia

(23). In 1915, Einthoven and Korteweg analyzed the indirect carotid pulse tracings obtained from patients with atrial fibrillation and noted that the height of the systolic deflections varied directly with the duration of the preceding cardiac cycle, and inversely with the height of the previous diastolic pressure. They concluded that the lower diastolic pressure associated with a longer diastolic interval permits the subsequent ventricular contraction to be more forceful than the higher diastolic pressure associated with a shorter interval (24). In the same year, in a study carried out on dogs with experimentally produced atrial fibrillation, Wiggers found that those ventricular contractions which followed preceding beats by a relatively short time interval were characterized by a lower systolic pressure and a more gradual systolic rise than those contractions which followed longer diastolic intervals (25). Katz and Feil, in a study of indirect carotid pulse tracings obtained from patients with atrial fibrillation, observed that both the height of each pulse as well as its duration are functions of the length of the preceding diastole (26). These studies were later extended by Buchbinder and Sugarman who recorded systemic arterial pressure pulses directly (27), and correlated the peak systolic arterial pressure, the pulse pressure, and the duration of ejection with the duration of the preceding cardiac cycle.

More recently, Dodge, Kirkham and King carried out a comprehensive study of ventricular dynamics in patients with atrial fibrillation, and pointed out that the arterial pulse pressure and systolic pressure are functions of the duration of the preceding R-R interval, and of the amplitude of the preceding pulse (28). Utilizing electrokymographic recordings to indicate changes in

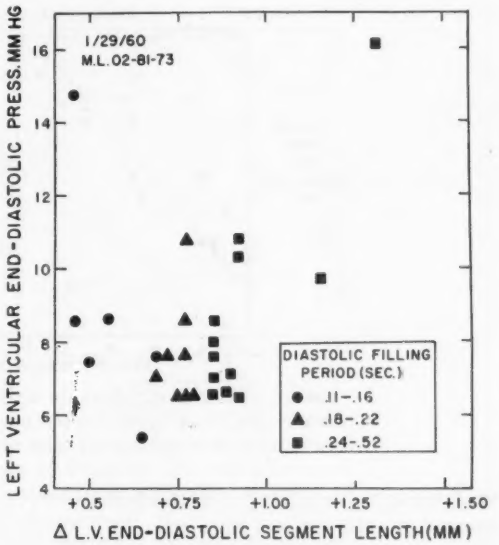

FIG. 9. RELATIONSHIP BETWEEN LV END-DIASTOLIC PRESSURE AND CHANGES IN END-DIASTOLIC SEGMENT LENGTH IN THE ONE PATIENT WITH SOME VERY BRIEF DIASTOLIC FILLING PERIODS.

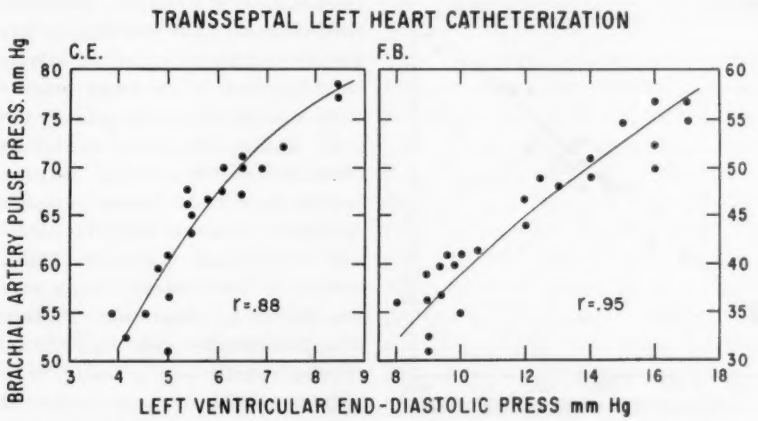

FIG. 10. TYPICAL RELATIONSHIP BETWEEN LV END-DIASTOLIC PRESSURE AND THE BRACHIAL ARTERY PULSE PRESSURE IN TWO PATIENTS STUDIED BY TRANSSEPTAL LEFT HEART CATHETERIZATION.

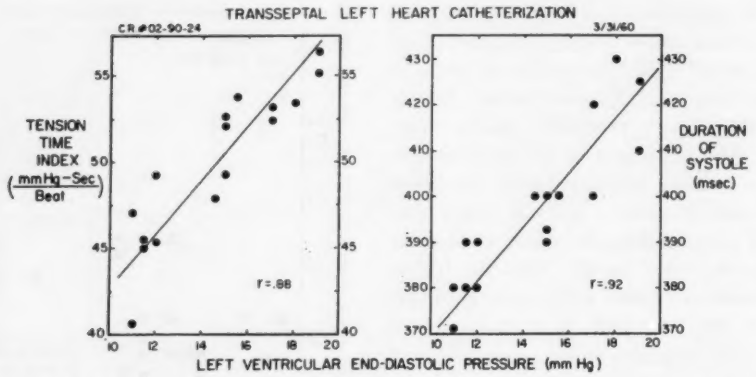

FIG. 11. TYPICAL RELATIONSHIP BETWEEN LV END-DIASTOLIC PRESSURE AND THE TENSION-TIME INDEX (LEFT PANEL) AND THE DURATION OF SYSTOLE (RIGHT PANEL). Data obtained at transseptal left heart catheterization.

ventricular volume, they confirmed the observations of Buchbinder and Sugarman (*27*) and also found an inverse correlation of the end-systolic "volume" with the height of the succeeding pressure pulse. Thus, a more forceful contraction resulted in a smaller end-systolic volume from which ventricular filling was initiated during the following diastole. These studies also provided evidence in support of the view that Starling's law of the heart operates in man.

In the present investigation, carried out on patients with mitral stenosis and atrial fibrillation, attention was focused on the relationship between the characteristics of each individual ventricular contraction and the segment length and pressure from which the contraction was initiated. It was evident from the studies carried out at the time of operation, that regardless of the manner in which the characteristics of ventricular contractions were assessed, *the left ventricular end-diastolic fiber length, as reflected by the left ventricular end-diastolic segment length, appeared to be a fundamental determinant of ventricular contraction.* Observations were then carried out on patients in the course of transseptal left heart catheterizations, in order to determine whether the close correlations between the characteristics of ventricular contraction and LVEDP, which were evident in the open-chest, anesthetized patients studied at operation, also exist in intact, closed-chest unanesthetized patients. The correlations between LVEDP and the characteristics of the subsequent ventricular contraction in the intact patients were similar to those observed in the patients studied at operation. Lauson, Bloomfield and Cournand have also demonstrated the essential features of Starling's law in patients in whom intrapleural and right ventricular pressures were recorded simultaneously (*29*). Although a regular cardiac rhythm was present in their patients, right ventricular filling was altered by respiration. A linear relationship was demonstrated between "effective" right ventricular end-diastolic pressure and the right ventricular systolic pressure of the subsequent beat.

Sarnoff and Berglund found no evidence of a descending limb in the ventricular function curves of open-chest dogs in which ventricular function

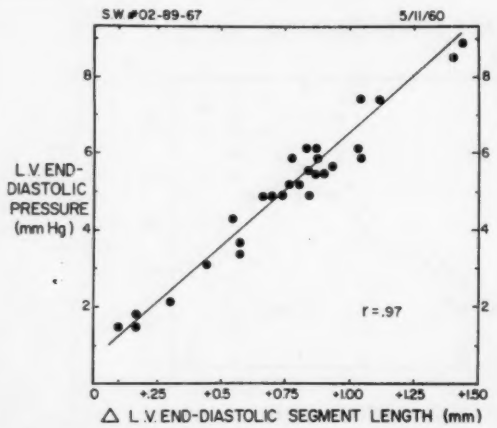

FIG. 12. TYPICAL RELATIONSHIP BETWEEN LV END-DIASTOLIC PRESSURE AND CHANGES IN END-DIASTOLIC SEGMENT LENGTH. Each point represents a single beat.

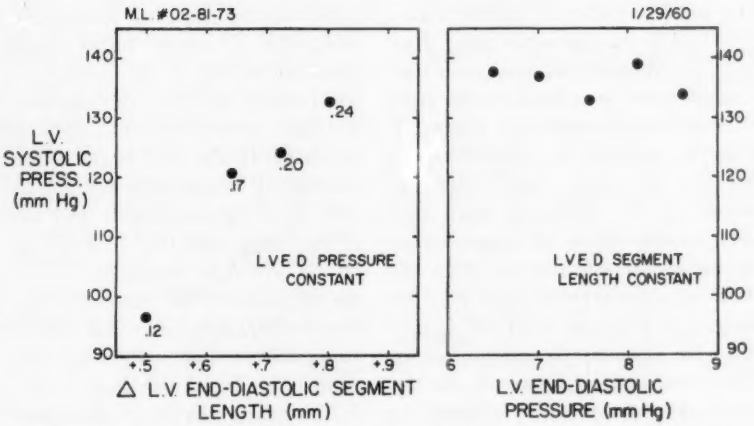

FIG. 13. DATA OBTAINED FROM THE SAME PATIENT WHOSE LV END-DIASTOLIC PRESSURE-SEGMENT LENGTH RELATIONSHIP IS PLOTTED IN FIGURE 9. The left panel illustrates the relationship between changes in LV end-diastolic segment length and LV systolic pressure in 4 beats, all of which had the same LV end-diastolic pressure. The figures to the right of each point represent the duration of the diastolic filling period in seconds preceding each beat. The right panel illustrates 5 other beats recorded from the same patient and depicts the relationship between LV end-diastolic pressure and LV systolic pressure. The LV end-diastolic segment length was identical in all 5 of these beats.

was not depressed (30). Similarly, in the present study, carried out in patients in whom left ventricular function was considered to be unimpaired, there was no instance of a descending limb of the curve relating LVEDSL or LVEDP and the four parameters utilized to characterize left ventricular contraction. It is possible that a descending limb would be evident if LVEDSL and LVEDP were elevated by an infusion. However, it is clear that at least within the range of end-diastolic pressures which obtain in the intact patient in the basal state, or in the anesthetized patient at the time of thoracotomy, the left ventricle appears to operate only on the ascending limb of its Starling curve.

The conclusion that the characteristics of each ventricular contraction are influenced by the preceding LVEDSL and LVEDP is based on a series of correlations which, of course, cannot prove the existence of a causal connection between LVEDSL or LVEDP and the characteristics of the subsequent contraction. The possibility that some other parameter which is the true determinant of ventricular contraction always correlates closely with LVEDSL or LVEDP cannot be excluded from these studies, nor from any of the classic observations which have led to the view that the length and tension existing in a muscle at the onset of contraction are important determinants of the character of the contraction (30–33). It is unlikely that the duration of the preceding diastole, per se, is the determinant of the characteristics of the subsequent ventricular contraction, since experiments performed on isolated cardiac muscle preparations indicate that the force of muscular contraction actually varies inversely with the duration of diastole (34, 35).

In the present investigation carried out on anesthetized, open-chest patients with mitral stenosis and atrial fibrillation, a linear relationship between LVEDP and LVEDSL was usually evident in the range through which these two parameters varied (Figure 12). It would therefore seem that under these conditions and in this range, the changes in effective EDP which occur in the course of a single series of beats may be used as an index of changes in end-diastolic segment length. However, it has long been suspected that changes in heart rate may alter the ventricular end-diastolic pressure-volume relationship (36). Indeed, in a recent study on the determinants of the left ventricular end-diastolic pressure-circumference relationship in the open-chest dog, it was observed

that at any given left ventricular end-diastolic circumference, the LVEDP was higher at rapid than at slower rates (37). When atrial fibrillation was induced, beats which were preceded by very brief R-R intervals were characterized by a greater EDP for any given end-diastolic circumference, than beats preceded by longer R-R intervals. These observations on the effects of heart rate, and similar ones on the effects of hypothermia, were thought to indicate that at the end of an abbreviated diastole ventricular relaxation may be incomplete, resulting in a greater LVEDP at any given left ventricular end-diastolic circumference (37). The data presented in Figure 9, on the one patient encountered in this investigation in whom the duration of diastole was sometimes strikingly abbreviated, confirm these experimental observations and extend them to man. It is of interest that in the present study, evidence was obtained in support of the view that the EDSL correlated better with the various indices of ventricular contraction than did the EDP (Figure 13), a position shared by Dodge and co-workers (28).

The observations reported herein were carried out on a series of consecutive beats which occurred in the course of less than 1 minute. It is possible that as a consequence of the beat-to-beat variations in baroreceptor stimulation, slight reflex alterations in myocardial contractility occurred (38), even during this short time period. It is possible that these alterations in contractility are responsible for the slight scatter which was observed between the LVEDSL or the LVEDP and the characteristics of the subsequent ventricular contraction. On the other hand, it would seem likely that substantial reflexly or humorally induced alterations in myocardial contractility occurred in those experiments in which infusions were utilized to study the applicability of Starling's law of the heart to man (2–6); these changes in contractility might well have obscured the underlying relationship between ventricular filling pressure and ventricular performance.

In a study recently carried out in this laboratory, the observation that the cardiac output and ventricular work fail to rise following the rapid transfusion of large volumes of blood into human subjects was confirmed (39); it was also demonstrated that the hypervolemia which was induced acutely was not accompanied by an augmentation of central blood volume. However, when the activity of the autonomic nervous system was reduced by means of ganglionic blockade with Arfonad (trimethaphan camphorsulfonate), augmentation of the total blood volume resulted in a substantial elevation of central blood volume as well as of cardiac output and ventricular work. It was suggested that in previous investigations on the effects of expansions of the blood volume, the applicability of Starling's law had not been tested adequately, since the presence of an intact autonomic nervous system may have actually prevented significant increases in the venous return to the heart. In other transfusion experiments, carried out on subjects under the influence of ganglionic blockade, it has been observed that ventricular stroke work varies directly with effective EDP (40). In this manner it has been possible to plot ventricular function curves (30) or "modified Starling" curves in man. In addition, the observations described in the present report indicate that under the conditions in which these observations were made and in which large changes in myocardial contractility do not occur, the characteristics of each individual ventricular contraction are closely related to the LVEDSL and LVEDP. Thus, both of these types of observations are consonant with the view that Starling's law operates in the human heart and is one of the important mechanisms which controls its function.

SUMMARY

The present investigation was designed to determine whether left ventricular end-diastolic segment length and end-diastolic pressure are important determinants of ventricular contraction. Systemic arterial pressure and effective end-diastolic pressure were measured at operation in 26 adult patients with rheumatic mitral valve disease and atrial fibrillation. Continuous alterations of the length of a segment of left ventricular muscle were recorded simultaneously in 13 of these patients by means of a mercury-filled resistance gauge sutured to the surface of the left ventricle. The variability of the duration of diastole resulted in beat-to-beat alterations of ventricular filling which resulted in variations of end-diastolic segment length and pressure. For each individual

beat the peak systolic ventricular pressure was utilized as an index of maximum tension developed; the area beneath the left ventricular pressure curve (tension-time index) was employed as a measure of the total tension developed. The systolic period indicated the duration of the tension state, and the systemic arterial pulse pressure was utilized to indicate relative changes in stroke volume. In every patient alterations in end-diastolic segment length correlated closely with all four parameters utilized to characterize ventricular function. In order to determine whether these relationships also exist in intact unanesthetized subjects, left ventricular end-diastolic pressure was determined by means of transseptal left heart catheterization in four patients. The correlations of end-diastolic pressure with systolic pressure, tension-time index, duration of systole, and arterial pulse pressure were similar to those obtained in the patients studied at operation. These results are consistent with the view that Starling's law of the heart operates in patients with mitral stenosis and atrial fibrillation on a beat-to-beat basis, and that end-diastolic segment length and end-diastolic pressure are important determinants of the characteristics of the subsequent ventricular contraction.

ACKNOWLEDGMENT

The authors gratefully acknowledge the cooperation of Drs. A. G. Morrow and N. S. Braunwald in permitting some of the observations which were carried out in the course of operation.

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ENHANCEMENT OF POTASSIUM–INDUCED ATRIOVENTRICULAR BLOCK BY TOXIC DOSES OF DIGITALIS DRUGS*

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In an attempt to evaluate the concept of K and digitalis antagonism, K was administered to patients with atrial fibrillation with slow ventricular rates due to digitalis. Some of these patients had associated ventricular ectopic rhythms. It was expected that by "unblocking" the effect of digitalis at the atrioventricular (A-V) node, the ventricular rate would increase. Contrary to expectations, the A-V delay was potentiated by the K, parallel with elimination of ventricular ectopic beats when the latter were present (1). This observation suggested that perhaps the concept of K and digitalis "antagonism" can be invoked only in respect to digitalis-induced arrhythmias. The latter observation goes back to 1918 when Dr. Loewi, in order to study the relation of Ca and K to digitalis, induced cardiac arrhythmias with strophanthin and described the effect of K on the arrhythmias in these words: "Schliesslich wurde gezeigt, dass so wie die Ca auch die Strophanthin wirkungen antagonistisch durch Kalium beeinflussbar sind" (Finally it was shown that strophanthin as well as Ca effect can be influenced antagonistically by potassium) (2). The value of potassium salts in abolishing ventricular ectopic beats due to digitalis in man was shown by Sampson, Alberton and Kondo (3) and confirmed by Enselberg, Simmons and Mintz (4). Bettinger and his associates, using intravenous KCl, found that the effect of K in depressing arrhythmias was equally as great whether or not patients received digitalis (5). The beneficial effects of K on atrial arrhythmia was first observed by Enselberg and co-workers (4) and was extensively studied by Lown and Levine (6). The transient nature of the depression of ventricular ectopic beats by K detracts somewhat from its therapeutic value (4, 7, 8). More significant, however, in view of the generally accepted and unqualified concept of K and digitalis "antagonism," has been the observation that while K eliminates ventricular ectopic beats, if given rapidly (about 2.0 mEq per minute for 1 to 2 minutes) it tends to prolong A-V conduction (4, 7, 8). Cardiac arrest has been observed during administration of potassium in patients with A-V block due to digitalis and there is evidence to suggest that the depressing effect of K on A-V conduction in man is more likely to occur after administration of digitalis (8–10).

The uniform but strikingly transient depression of digitalis-induced arrhythmia by K has been recently observed in animal study (11). It has also been shown that in animals poisoned with large doses of digitalis and subsequently infused with K, the average amount of K necessary to kill the animal was less in the digitalis-intoxicated than in comparable control animals (12, 13).

In view of the largely transient nature of the effect of K on ectopic arrhythmias with depression of A-V and intraventricular conduction and lack of evidence that K counteracts any other parameter of digitalis toxicity, the concept of specific K and digitalis "antagonism," as it applies to effects other than arrhythmias, deserves further investigation. The purpose of the present study was to evaluate, in dogs, the effect of K on A-V conduction before and after administration of toxic doses of digitoxin.

MATERIAL AND METHOD

A total of 53 experiments, 42 without digitoxin and 12 with digitoxin, were performed on 9 mongrel dogs weighing 9.8 to 15 kg, over a period of 9 months. The animals were anesthetized with 30 mg of Na pentobarbital per kg of body weight given intraperitoneally. In all but 10 experiments (Table I, Experiments 75, 76, 82, 83, 92, 162, 103, 128, 123 and 156), the administration of K was preceded by intravenous injection of acetyl strophanthidin (Lilly). An ampule containing 0.6 mg of the drug was

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diluted with 9 cc of saline and 1 cc (0.06 mg) of the solution was injected every 30 seconds. Because the peak action of acetyl strophanthidin may not become manifest for 5 to 10 minutes after the drug is given, the administration was usually discontinued when occasional ectopic beats appeared, usually A-V nodal or ventricular escape beats or slow escape rhythm. The latter was invariably followed by a tachycardia usually identifiable, because of fusion (combination) beats, as being ventricular in origin. Within 2 to 4 minutes after appearance of the tachy-

TABLE I

Amount of acetyl strophanthidin and potassium required to cause arrhythmia or A-V block in dogs before and after the administration of digitoxin

		Before digitoxin				After digitoxin				
Dog no.	Exp. no.	A.S.* to arrhythmia	Amount of K to A-V block		Plasma K at A-V block	Digitoxin†	A.S. to arrhythmia	Amoung of K to A-V block		Plasma K at A-V block
			Total					Total		
		mg	*mEq*	*mEq/kg*	*mEq/L*	*mg/kg*	*mg*	*mEq*	*mEq/kg*	*mEq/L*
29	74	0.72	16.5	1.48	7.8					
	75		12.3	1.10	7.5					
	76		15.7	1.42	7.3					
	82		10.5	0.94	7.4					
	86					0.43	0.78	6.4	0.57	7.1
33	79	0.96	16.8	1.09	8.2					
	81	0.78	16.5	1.07	9.4					
	83		20.2	1.31	8.3					
	84					0.33	0.42	6.0	0.38	6.3
34	90	1.02	15.0	1.22	7.3					
	92		23.2	1.90	8.6					
	124	1.08	18.7	1.53	8.6					
	139	1.02	15.7	1.28	8.5					
	140	1.02	17.3	1.41	9.0					
	146	0.96	22.5	1.84	8.2					
	162		11.5	0.94	6.5					
	93					0.40	0.84	9.8	0.80	6.7
	148					0.45	0.36	10.5	0.86	6.8
35	91	1.14	21.0	2.05	9.0					
	95†	1.02	13.1	1.28	8.2					
	125	1.20	16.8	1.64	8.9					
	137	1.02	12.0	1.17	7.7					
	138	0.96	10.5	1.02	8.6					
	147	1.14	11.3	1.10	7.4					
	163	0.84	8.2	0.80	7.2					
	96					0.41	0.72	4.8	0.47	6.5
	149					0.49	0.24	4.6	0.45	7.8
36	101	1.14	21.0	1.72	8.5					
	103		18.8	1.54	7.8					
	126	0.90	15.7	1.28	7.4					
	128		17.3	1.41	8.3					
	104					0.33	1.14	5.2	0.42	7.1
38	123		19.5	1.57	8.7					
	127	1.20	19.8	1.59	7.3					
	131	1.20	16.5	1.33	7.5					
	150	0.96	18.7	1.50	9.0					
	164	0.96	17.2	1.38	7.1					
	132					0.40	0.72	5.6	0.45	6.3
	152					0.42	0.78	12.7	1.02	7.3
39	129	1.32	17.2	1.84						
	133	1.38	12.7	1.36	8.6					
	142	1.38	12.8	1.37	8.7					
	144	1.20	9.8	1.05	8.0					
	136					0.50	0.90	4.5	0.48	7.5
40	134	1.56	26.3	1.86	9.9					
	143	1.62	21.0	1.48	9.5					
	145	1.20	19.0	1.34	11.3					
	156		18.0	1.27	9.2					
	160	0.90	15.7	1.11	8.4					
	135					0.41	1.20	4.5	0.31	6.9
41	151	1.32	19.5	1.43	9.3					
	165	1.20	19.9	1.46						
	153					0.35	0.90	12.0	0.88	7.3

* A.S. = acetyl strophanthidin (Lilly).
† Not to exceed 5.0 mg in any animal.

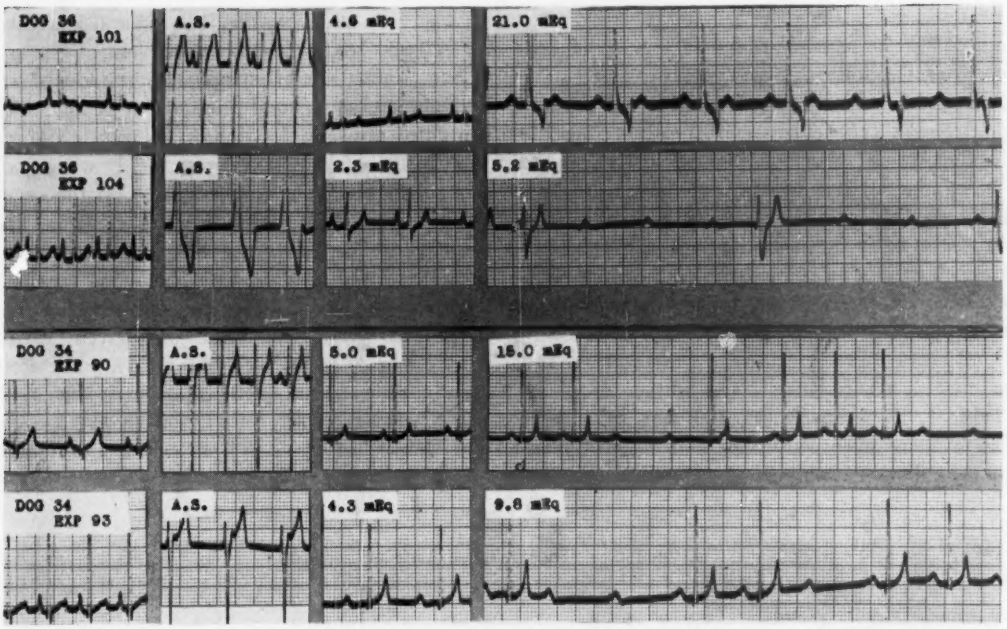

FIG. 1. EFFECT OF POTASSIUM BEFORE AND AFTER DIGITOXIN INTOXICATION IN ANIMALS 36 AND 34. This experiment is characteristic of most of those conducted. Experiment 101, Dog 36, was performed before digitoxin intoxication. The short strip in the upper lefthand corner represents control tracing; the strip marked A.S. indicates the ventricular arrhythmia induced by acetyl strophanthidin. The latter was abolished with 4.6 mEq of K, and after 21 mEq of K was given a higher degree A-V block appeared. Experiment 104, performed after digitoxin intoxication, shows a similar sequence of events with the exception that the acetyl strophanthidin-induced arrhythmia was abolished with 2.3 mEq of K, with intraventricular block and a high degree of A-V block being obvious after only 5.2 mEq of the cation. In Dog 34, before digitoxin intoxication (Experiment 90), the acetyl strophanthidin-induced arrhythmia was abolished with 5 mEq of K and block appeared after 15 mEq of the ion was given. Following digitoxin intoxication (Experiment 93) the acetyl strophanthidin ventricular tachycardia was abolished with 4.3 mEq and block appeared after 9.8 mEq of K was infused.

cardia, isotonic solution (155 mEq per L) of buffered K phosphate in distilled water was injected at a rate of 1.10 to 1.23 mEq per minute until A-V block appeared. A flow meter was used to assure a constant flow. The rate of flow as determined from the flow meter was checked against the rate determined from the volume of solution used and the time required for its infusion. In 12 experiments, following the infusion of K, the animals were given intramuscularly 0.33 to 0.50 (average 0.40) mg of digitoxin per kg of body weight, the total dose not exceeding 5.0 mg. Forty-eight hours later the dogs were returned to the laboratory and reinfused with K in the manner described above.

The entire experiment was monitored with an oscilloscope. All cardiograms were recorded with direct writing equipment using standard lead II connections.

Venous plasma K was determined with a Beckman flame photometer before the start of infusion, during the infusion, and at appearance of A-V block. In a number of experiments, Na, Cl, Ca and O_2 saturation were determined before and after administration of acetyl strophanthidin and K.

Isotonic solutions of KCl and Na phosphate were used as control fluids. KCl was injected to A-V block and Na phosphate was given in amounts twice or three times that of the previously administered K phosphate.

RESULTS

The amount of digitoxin administered (0.33 to 0.50 mg per kg) was considered sufficiently large to cause intoxication. On two occasions the animals failed to eat or drink and had diarrhea. The maximal weight loss during any 48 hour period was 0.4 kg. Ventricular arrhythmia was observed on two occasions only. The P-R interval remained unchanged in seven experiments; in three the duration increased from 0.80 to 0.10, in one from 0.12 to 0.14 and in another from 0.09 to 0.13. Significant degree of ST depression was observed on one occasion only. The preinfusion ventricu-

lar rates in animals not given digitoxin varied from 75 to 200 beats per minute for an average of 126, and after digitoxin the control rate varied from 136 to 206 for an average of 162 beats per minute. The average ventricular rates shortly before the K-induced block appeared were 127 and 119 in the control and digitoxin-intoxicated animals, respectively. Increased sensitivity to acetyl strophanthidin after administration of digitoxin was observed in nine of the experiments, with the reduction of the drug required to produce ventricular tachycardia being from 10 to 25, from 25 to 50, and over 5 per cent in two, five and three experiments, respectively. On three occasions (Table I, Experiments 86, 104 and 135) there was no increase in sensitivity to acetyl strophanthidin, although in two of the experiments (Experiments 86 and 104) the P-R interval increased by 44.0 and 16.0 per cent, respectively.

In all animals acetyl strophanthidin-induced arhythmia was suppressed promptly by K and, with continued infusion, A-V block followed (Figures 1, 2). In a few experiments, the P waves, although distorted but clearly visible on the oscilloscope, were poorly identifiable on the EKG. The amount of K necessary to produce A-V block was greatly reduced in all animals, following administration of digitoxin. The total amount of K administered before digitoxin was given varied from 8.2 to 26.3 (average 16.6 mEq), while after digitoxin the amount of the cation varied from 4.5 to 12.7 (average 7.2 mEq), for an average reduction of 9.4 mEq of 56.6 per cent in the amount of cation required to induce A-V block. The

FIG. 2. SINO-ATRIAL ARREST AND BLOCK IN TWO DOGS. In Dog 35, Experiment 91 was performed before, and Experiment 96 after digitoxin intoxication. In Experiment 91 the ventricular tachycardia was abolished after 4.9 mEq of K and A-V block appeared with 21.0 mEq; in Experiment 96 these results were accomplished after 1.5 and 3.6 mEq, respectively. In Dog 39, Experiment 133 was performed before, and Experiment 136 after administration of digitoxin. The amount of K necessary to abolish acetyl strophanthidin-induced ventricular tachycardia and to produce A-V block was 7.4 and 12.7 mEq in Experiment 133, and 4.5 in Experiment 136.

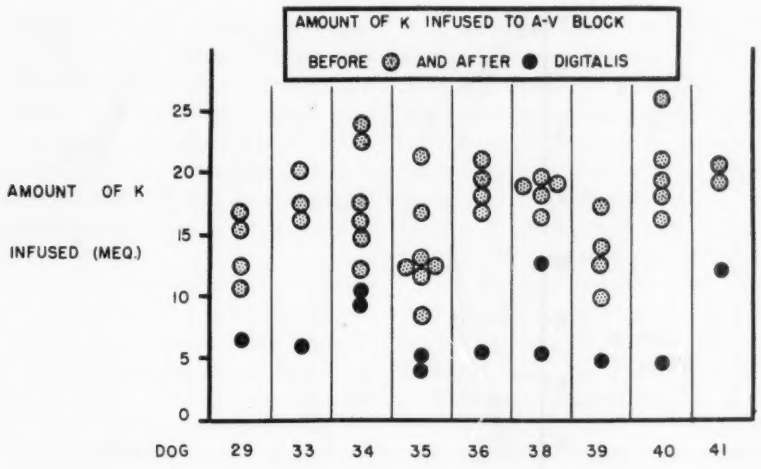
AMOUNT OF K INFUSED TO A-V BLOCK
BEFORE AND AFTER DIGITALIS
AMOUNT OF K
INFUSED (MEQ.)
25
20
15
10
5
0
DOG 29 33 34 35 36 38 39 40 41
AV. K BEFORE DIG. 13.7 17.8 17.7 13.2 18.2 18.3 13.1 20.0 19.7
AV. K AFTER DIG. 6.4 6.0 10.1 4.7 5.2 9.1 4.5 4.5 12.0

FIG. 3. AMOUNT OF K NECESSARY TO PRODUCE A-V BLOCK BEFORE (STIPPLED CIRCLES) AND AFTER (SOLID CIRCLES) DIGITOXIN INTOXICATION IN THE NINE ANIMALS STUDIED. The number of the animal is shown on the abscissa and the amount of K (in millequivalents) necessary to produce A-V block is shown on the ordinate. The average amounts of K (in milliequivalents) needed to cause A-V block in the control and in the digitoxin-intoxicated animals are given below the graph. It is obvious from these data that the amount of K necessary to produce A-V block after digitoxin administration was markedly reduced.

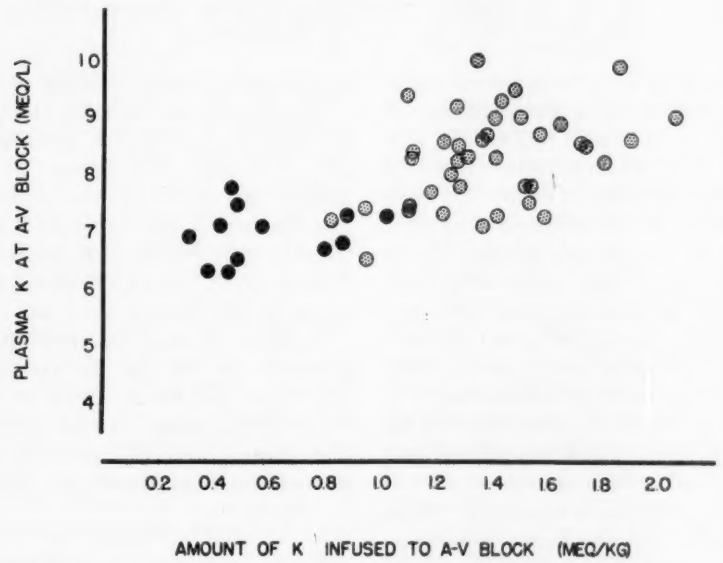

FIG. 4. PLASMA LEVEL OF K AT A-V BLOCK (ORDINATE) AND THE AMOUNT OF K (ABSCISSA) NECESSARY TO PRODUCE THE BLOCK, BEFORE (STIPPLED CIRCLES) AND AFTER (SOLID CIRCLES) ADMINISTRATION OF DIGITOXIN.

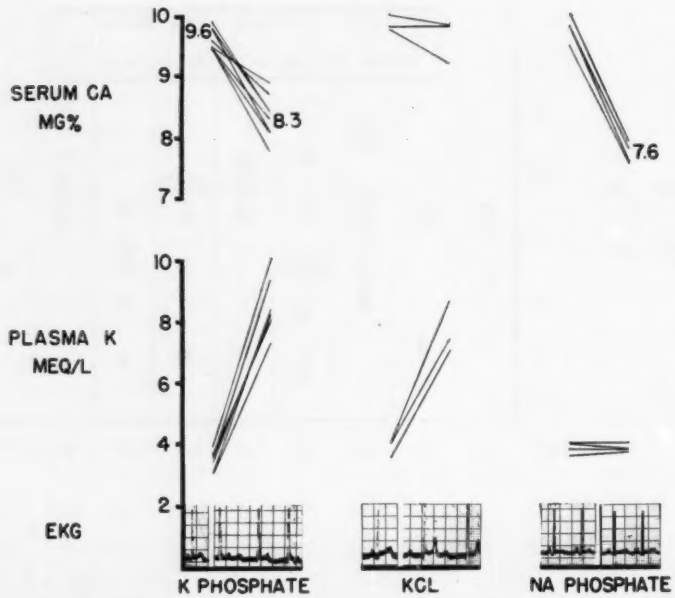

FIG. 5. COMPARISON OF THE EFFECT OF K PHOSPHATE, NA PHOSPHATE AND KCL ON THE SERUM CA, PLASMA K AND A-V CONDUCTION. The three different solutions are listed on the abscissa. On the ordinate are represented serum Ca, plasma K, and the EKG consisting of a short control strip and tracing at termination of infusion. Sodium phosphate, although administered in large amounts and causing most marked depression of serum calcium, did not affect either the plasma K or A-V conduction. On the other hand, KCl, although not affecting the serum calcium, caused a rise in plasma potassium and produced an A-V block similar to one seen with K phosphate.

amount of K per kg of body weight necessary to produce block before digitoxin administration varied from 0.80 to 2.05 (average 1.37 mEq); after digitoxin intoxication the dose varied from 0.38 to 0.88 (average 0.59 mEq) (Figure 3). The block lasted longer in the digitoxin-intoxicated animals than in the control experiment. In addition sino-atrial (SA) blocks and arrest were seen commonly in the digitoxin-intoxicated animals but were rare in dogs not given digitoxin (Figure 2). The average plasma K level at which A-V block was produced before administration of digitoxin was 8.5 mEq per L; after digitoxin the average plasma K level at block was 6.9 mEq per L (Figure 4). Under the hypothesis of no real difference both the changes in amount as well as in plasma level before and after digitoxin were significant on less than 1 per cent level. The amount of acetyl strophanthidin, K phosphate, digitoxin, and the plasma level of K at A-V block used in each of the 53 experiments are given in Table I.

The effect of KCl on EKG and plasma K level was the same as that of K phosphate. Na phosphate, although administered in much larger amounts and causing a more pronounced depression of serum Ca than either KCl or K phosphate, failed to alter the the P-R interval (Figure 5). Plasma Na, Cl, and O_2 saturation showed no consistent or quantitatively significant change.

Omission of acetyl strophanthidin in nine experiments did not alter the amount of K necessary to produce block. None of the animals in this particular group of nine received digitoxin. The amounts were 16.7 and 16.6 mEq with and without acetyl strophanthidin, respectively.

DISCUSSION

A-V block, as a constant manifestation of experimentally-induced hyperkalemia is rarely referred to in the literature. It was surprising to

us, therefore, to find A-V block with such regularity. We feel that the reproducibility of A-V block was probably due to the rate of infusion, for with slower rates disappearance of P waves and with higher rates cardiac standstill preclude the recognition of A-V block. S-A arrest and S-A block were observed when K was given to digitoxin-intoxicated animals, but were not seen in the control dogs.

The A-V block, S-A arrest and block are doubtless due to K. The results of control studies using isotonic KCl and Na phosphate with frequent determinations of Na, Cl, Ca and O_2 saturation are in keeping with this conclusion. There is also little doubt that the disturbance of impulse formation and conduction due to K was greatly enhanced by previous administration of digitoxin. The depressing effect of both digitoxin and K on spontaneous activity of normal pacemakers, as well as on the refractory period and speed of impulse transmission of the A-V conduction system explains the relative ease with which S-A arrest, S-A and A-V blocks appear when K is administered to a digitoxin-intoxicated dog (14–16). The exact mechanism by which the effect of K is "enhanced" by digitoxin so that the conduction disturbances not only occur with less K but also at a lower plasma level is not clear. In the light of our present knowledge a combination of three possible mechanisms suggests itself: *1*) the egress of K from the cells in the presence of a normal extracellular K increases the extra- to intracellular ratio of the cation; *2*) the interference with intracellular transfer of K by toxic doses of digitoxin tends to maintain this increased ratio; *3*) the consequently more rapid rise of plasma K in the animal given digitoxin produced a critical plasma level with less K. This more rapid rise was observed by Williams, Spirakis and Keyl (17) in strophanthin-poisoned dogs. Although we were unable to detect significant loss of water or change of plasma Na, Cl and O_2 saturation, there is little doubt that disturbance of electrolytes other than K, change in pH, O_2 saturation, and body water that may result from digitalis intoxication, can and possibly may have added to the increased sensitivity to K observed in our animals. It seems reasonable to assume that given a condition in which there exists an abnormally high extra- to intracellular ratio of potassium and in which the K is prevented from entering the cells, less K is required to produce a critical plasma level. Furthermore, because of intracellular depletion of the cation the critical ratio between extra- and intracellular concentration of K will be reached at lower plasma levels of K than in the nondigitalized animals. A comprehensive review of the cellular basis of digitalis action can be found in a recent paper by Hajdu and Leonard (18).

The doses of digitoxin used varied from 0.33 to 0.50 mg per kg with an average dose of 0.40 mg per kg and did not exceed 5.0 mg in any one animal. Based on observation of the animals, P-R changes, and appearance of ventricular arrhythmias, we felt that in 10 out of 12 episodes of digitalization the animals were under the influence of less than 50 per cent of the lethal dose of digitoxin (15). Why more severe signs of digitoxin intoxication were not evident in our animals is difficult to answer with any degree of certainty. It is possible that the absorption of digitoxin from the site of injection was incomplete or perhaps the excretion in the first 24 to 48 hours was rapid due to large amounts of the initially given drug. It was also noted that the same animals, when repeatedly given the same amount of digitoxin, did not manifest uniform EKG changes. Any estimate of digitoxin intoxication on the basis of EKG changes alone may be misleading.

In our experiments omission of acetyl strophanthidin did not affect the amount of K that was necessary to produce A-V block. The nine experiments, however, in which acetyl strophanthidin was omitted comprise a group too small to make these observations valid. In addition the design of our experiments would not permit a study of transient effects of K flux attending the administration of acetyl strophanthidin. Regan, Talmers and Hellems (19) and more recently Cairns, Love and Burch (20), in experiments specifically designed to study the effect of acetyl strophanthidin on K flux, have shown that the drug causes an efflux of K and blocks re-entry of K into the cell. According to Cairns and his associates the effect lasts about 9 to 12 minutes.

It is possible that potentiation of the vagal effect of digitoxin contributed to S-A arrest and block and A-V conduction delay. However, a major effect is unlikley because of use of pentobarbital as the anesthetic agent. Barbiturates have

been shown to depress reflex activity. Moe and Mendez (15) administered digitoxin to dogs under pentobarbital anesthesia and observed that the effects of the drug were the same both in the denervated and intact animal. Furthermore, administration of 5 mg of atropine intravenously to some of our animals, not in this study, did not prevent the appearance of A-V block during infusion of K. The role of the *in situ* production of acetylcholine that would not be inhibited by atropine is difficult to evaluate.

The control heart rate was consistently higher in the digitoxin-intoxicated animal, but the rate shortly before appearance of A-V block was the same or slightly lower in the intoxicated dogs. It is doubtful, therefore, that the ventricular rate per se contributed significantly to the enhanced depression of A-V conduction observed in the digitoxin-intoxicated animals.

The present day concept of digitalis and K "antagonism" seems to be based only on the rather nonspecific depressing effect of K on ectopic rhythms. There is no doubt, from the studies of Lown and Levine (21), that an elevated plasma K protects against, and lowered plasma K enhances ventricular ectopic beats. On the other hand, experimental evidence, suggesting that depletion of body K without altering plasma K "sensitizes" to digitalis, is less convincing (22, 23). Regardless of the state of body K, there is very little, if any, evidence that K "antagonizes" any features of digitalis activity other than digitalis-induced arrhythmia. There is every reason to believe that in the presence of normal stores of K and perhaps in some K-deficient states, elimination of ectopic rhythms may in many cases be related simply to a rise in extracellular K. This impression is supported by the frequently transient nature of depression of ventricular ectopic beats in man, often paralleled by slowing of A-V conduction (4, 7, 8). Experimentally produced ventricular arrhythmia can be abolished by raising plasma K to 6 mEq per L but recurs when the K level drops below 6 mEq per L (10). We have observed similar behavior of ectopic beats in respect to plasma K level in humans. It is difficult to conceive that 6 to 20 mEq of K would exert a beneficial effect on an arrhythmia through any mechanism than perhaps by a transient elevation of the extracellular levels of the cation (8). Failure of arrhythmia to reappear after the plasma level of K returns to normal after once being acutely elevated does not necessarily imply, as some have suggested, that the effect of K must be due to intracellular shift of the cation with a change of intra- and extracellular ratio.

The fact that K depresses A-V conduction (4, 7–10) and that this depression is potentiated by digitoxin (10, 12, 13) may be of clinical significance and would imply that, in the presence of impaired A-V conduction, the cation should be given with great caution. This does not mean that K should be withheld in face of digitalis-induced arrhythmia. There is uniform agreement that K is an excellent anti-arrhythmia drug and its use may mean the difference between life and death. Ectopic rhythms are affected by considerably smaller amounts of K than those required to affect A-V conduction. This fact allows for a significant "margin of safety" when the cation is used to control arrhythmias due to digitoxin intoxication.

SUMMARY

1. Buffered isotonic K phosphate was administered 42 times to nine dogs before digitalization. An average of 16.6 mEq of the cation was necessary to produce atrioventricular (A-V) block.

2. The same procedure was carried out 12 times after administration of toxic doses of digitoxin. In this group an average amount of only 7.2 mEq was necessary to produce A-V block, and sinoatrial block and arrest were frequent in this group.

3. Infusion of K into animals intoxicated with digitoxin produced A-V block not only with less K, but also at a lower plasma level. There was also a steeper rise of plasma K level in this group of experiments.

4. These studies suggest that K should be given with caution to digitalis-intoxicated patients in whom the intoxication is manifested by abnormalities of A-V conduction.

ADDENDUM

The experimental data collected in course of infusion of K either before or after administration of acetyl strophanthidin is part of a large series which constitute our laboratory control and is repeatedly used as a standard against which the response to potassium, when other factors (e.g., additional digitoxin) are varied, is meas-

ured. These control data therefore are an integral part of other studies dealing with the subject of potassium and digitalis antagonism.

ACKNOWLEDGMENT

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ELECTROPHORETIC COMPONENTS OF THE HEMOGLOBIN OF RED CELL MEMBRANES *

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Erythrocyte membranes prepared by repeated washings with water have been shown by Anderson and Turner (1) to contain relatively constant amounts of hemoglobin. The hemoglobin of lyophilized erythrocyte membranes represented about 3 per cent of the total erythrocyte hemoglobin and between 49 and 57 per cent of dry weight of the membranes (1). No differences between the membrane hemoglobin and the water-soluble hemoglobin obtained by the lysis of the whole erythrocytes have been noted in spectrophotometric studies when both were converted to cyanmethemoglobin (2). However, if a part of the hemoglobin is indeed bound to the membrane it might exhibit other properties distinguishing it from the remainder of the hemoglobin of the red cell.

The present studies were designed to examine further whether the hemoglobin attached to membranes differs from the hemoglobin obtained in watery solution after lysis of erythrocytes with water and toluene by the method of Drabkin (3), or from the water-soluble hemoglobin obtained by lysis of erythrocytes with 12 volumes of water.

MATERIALS AND METHODS

Blood samples were taken in acid citrate dextrose (ACD) solution and the erythrocytes were washed 4 times with normal saline. An aliquot of washed erythrocytes was lysed with water and toluene by the method of Drabkin (3), and a clear solution of hemoglobin was obtained after centrifugation at 15,000 × G for 40 minutes. This hemoglobin preparation, designated "hemolysate hemoglobin," was converted to cyanmethemoglobin or carbonmonoxyhemoglobin for analyses. Comparative electrophoretic studies of hemolysate hemoglobin prepared by lysis with water and toluene and of water-soluble hemoglobin obtained by lysis of the washed erythrocytes with 12 volumes of cold water revealed no significant differences in the relative amounts of the major and minor hemoglobin components. Therefore, either preparation was utilized for comparison with the membrane pigment.

After lysis of the erythrocytes with 12 volumes of cold water, washings of the membranes with cold water were continued until no color could be observed in the water; the red cell membranes were still distinctly reddish in color. To remove the hemoglobin from the erythrocyte membranes two different procedures were employed. In the first procedure, cyanmethemoglobin was prepared by the addition of 2 to 3 volumes of 50 per cent urea in Drabkin's diluent [1] (4) to the wet membranes. The mixture was agitated for 5 to 10 minutes and centrifuged at 15,000 × G for 40 minutes. After centrifugation, the reddish precipitate of stroma was discarded. The clear supernatant solution of cyanmethemoglobin was dialyzed with stirring against at least 500 volumes of cold distilled water, with several changes, and centrifuged again before analyses were carried out. Cyanmethemoglobin concentrations of 0.2 to 0.3 g per 100 ml were obtained by this method, and further concentration to 1 g per 100 ml could be achieved in a Toribara ultrafiltration apparatus. When membrane hemoglobin prepared by this procedure was compared with hemolysate hemoglobin, the hemolysate hemoglobin was diluted with urea-Drabkin's diluent and dialyzed in the same manner. In a second procedure for the preparation of membrane hemoglobin the carbonmonoxy pigment was obtained. The lysed erythrocytes were exposed to carbon monoxide and the wet membranes collected by centrifugation. The packed wet membranes were then homogenized in an equal volume of cold veronal buffer (pH 8.6, ionic strength 0.04) for 2 to 4 minutes in a multi-mixer (Lourdes). The mixture was centrifuged and the clear supernatant solution of pigment was again exposed to carbon monoxide. Dialysis was omitted; water-soluble carbonmonoxyhemoglobin was compared electrophoretically with the preparation of membrane hemoglobin.

Electrophoretic analyses of the hemoglobin preparations were carried out by the starch granule technique of Kunkel, Ceppellini, Müller-Eberhard and Wolf (5). Hemolysate or water-soluble and membrane hemoglobin preparations of approximately the same concentration

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[1] Drabkin's diluent solution consists of 1.0 g $NaHCO_3$, 50 mg KCN, 200 mg $K_3Fe(CN)_6$, and distilled water to 1 L.

were run simultaneously on the same starch block, and the concentration of pigment in eluates was determined with a Beckman DU spectrophotometer. Duplicate or triplicate analyses were carried out for each preparation. Electrophoretic separations were also carried out on starch gels by the method of Smithies (6) at 4° C, using 180 v for 16 hours. The gels were stained with both benzidine and amido-black.

RESULTS

Human subjects without known hemoglobin abnormalities. Hemolysate or water-soluble hemoglobin and membrane hemoglobin were prepared from the erythrocytes of seven normal subjects and analyzed electrophoretically on starch granules (5) and starch gels (6). Normal human hemoglobin contains at least three electrophoretically distinct components: the slow moving minor component, hemoglobin A_2, which includes 1.5 to 3.5 per cent of the total pigment; the rapid minor component, hemoglobin A_3, which may comprise approximately 4 to 12 per cent of the total; and the main component, hemoglobin A_1 (5, 7). All three of these components were demonstrated by both starch block and starch gel electrophoresis of normal membrane hemoglobin prepared by either procedure. Concentrations of membrane hemoglobin of 0.2 g per 100 ml were sufficient to demonstrate both minor components on starch gels. However, for quantification of minor components on starch granules, concentrations of membrane hemoglobin of at least 0.5 to 1 g per 100 ml were desirable. When membrane hemoglobin was prepared by mechanical homogenizing in veronal buffer, carbonmonoxyhemoglobin concentrations sufficient for quantification on starch granule electrophoresis were achieved. Quantitative estimations (as per cent of total hemoglobin) for the electrophoretically slow (A_2) component of water-soluble and membrane hemoglobin of seven normal subjects are recorded in Table I. Values for hemoglobin A_2 in water-soluble hemoglobin ranged from 1.2 to 2.7 per cent. These values are within normal range for hemoglobin A_2 estimations on hemolysate hemoglobin (5). The electrophoretically slow component of the membrane pigment ranged from 4 to 10 per cent of the total hemoglobin in the membrane. The proportion of electrophoretically slow component in the membrane hemoglobin was therefore approximately three to four times that in the water-soluble pigment of these normal subjects.

TABLE I

Per cent of electrophoretically slow (A_2) hemoglobin in water-soluble and membrane hemoglobin

Normal subjects	Per cent A_2 hemoglobin in	
	Water-soluble hemoglobin*	Membrane hemoglobin†
H.R.	2.7	7.6
G.O.	1.7	7.0
J.P.	1.2	4.1
M.R.	1.8	6.6
L.D.	1.5	5.1
M.M.	2.2	10.3
P.M.	2.0	6.9
Median	1.8	6.9

* Carbonmonoxyhemoglobin prepared by lysis of erythrocytes with 12 volumes of water.

† Carbonmonoxyhemoglobin prepared by mechanical homogenizing of membranes in veronal buffer.

TABLE II

Per cent of abnormal major hemoglobin component in hemolysate (or water-soluble) hemoglobin and in membrane hemoglobin

Subjects	Hemoglobins	Percentage of designated hemoglobin in	
		Hemolysate hemoglobin	Membrane hemoglobin
E.R.	S + A	40 (S)*	62 (S)†
A.S.	S + A	41 (S)*	66 (S)†
E.G.	S + A	39 (S)*	50 (S)†
P.W.	S + A	42 (S)*	65 (S)†
S.G.	S + A	38 (S)*	51 (S)†
E.W.	S + A	31 (S)*	50 (S)†
M.M.	S + A	39 (S)*	60 (S)†
M.A.	S + A	38 (S)*	53 (S)†
C.H.	S + A	39 (S)*	63 (S)†
A.P.	S + A	26 (S)*	64 (S)†
Ca.L.	S + A	33 (S)‡	50 (S)§
Ce.L.	S + A	44 (S)*	80 (S)§
	S + A	42 (S)‡	73 (S)§
G.S.	S + A	33 (S)‡	60 (S)§
Average	S + A	37.5 (S)	60.5 (S)
Range	S + A	26–44 (S)	50–80 (S)
C.S.	S + C	45 (C)‡	72 (C)§
T.M.	S + C	48 (C)‡	80 (C)§
L.I.	S + C	45 (C)‡	75 (C)§
Average	S + C	46 (C)	72 (C)
A.K.	C + A	34 (C)‡	86 (C)§
R.I.	C + A	21 (C)‡	50 (C)§
Average	C + A	28 (C)	68 (C)

* Hemolysate hemoglobin prepared by lysis of erythrocytes with water and toluene.

† Cyanmethemoglobin prepared with urea-Drabkin's diluent.

‡ Water-soluble hemoglobin prepared by lysis of erythrocytes with 12 volumes of water.

§ Carbonmonoxyhemoglobin prepared by homogenizing membranes in veronal buffer.

Subjects with electrophoretically abnormal hemoglobin. The results of starch granule electro-

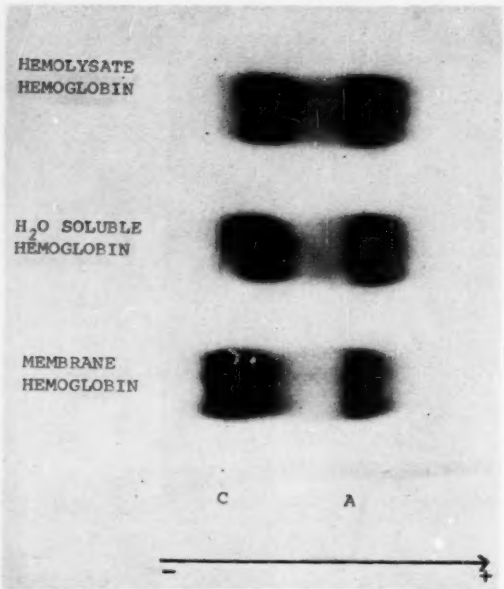

FIG. 1. STARCH GEL ELECTROPHORESIS OF HEMOLYSATE, WATER-SOLUBLE, AND MEMBRANE HEMOGLOBIN FROM A SUBJECT WITH HEMOGLOBIN C TRAIT. Concentrations of all preparations were 0.5 g per 100 ml. Electrophoresis in borate buffer at pH 8.8 for 16 hours; benzidine stain. Note the greater proportion of hemoglobin C in the membrane preparation.

phoretic analyses of hemolysate or water-soluble hemoglobin and of membrane hemoglobin in 18 subjects with heterozygous or compound states for the abnormal hemoglobins are recorded in Table II. In 13 subjects with sickle cell trait, values for hemoglobin S in the hemolysate or water-soluble hemoglobin ranged from 26 to 44 per cent; these values are similar to those reported by other investigators (8, 9). In contrast, the values for hemoglobin S in membrane hemoglobin ranged from 50 to 80 per cent of the total membrane pigment. Elevated values for hemoglobin S in membranes were found in both carbonmonoxy and cyanmethemoglobin preparations.

Studies of the erythrocytes of three individuals with sickle cell-hemoglobin C disease disclosed 45 to 48 per cent hemoglobin C in the water-soluble pigment and 70 to 75 per cent hemoglobin C in the membrane hemoglobin. In two individuals with hemoglobin C trait, larger proportions (50 to 60 per cent) of the abnormal hemoglobin were found in the membranes than in the hemolysate hemoglobin (21 and 34 per cent). Starch gel electrophoretic analyses of these hemoglobin preparations demonstrated clearly the relative increase in electrophoretically slow components in the membrane pigment (Figure 1).

In individuals with sickle cell trait or hemoglobin C trait, the abnormal hemoglobin was found to be increased in the membrane pigment as compared with the hemolysate hemoglobin, in every case. In the three individuals with sickle cell-hemoglobin C disease, the proportion of the electrophoretically slower hemoglobin C was found to be increased in the membrane preparations.

Patients with thalassemia minor. Four patients with hematological findings of thalassemia minor, including elevated values of hemoglobin A_2 in the hemolysate hemoglobin, were studied. The results of starch granule electrophoretic analyses of the water-soluble hemoglobin and of membrane carbonmonoxyhemoglobin are recorded in Table III. An increased proportion of hemoglobin A_2 was observed in the membrane hemoglobin as compared with the water-soluble pigment. The average value for water-soluble hemoglobin A_2 was 5.4 per cent, while hemoglobin A_2 accounted for 11 per cent of the membrane pigment.

DISCUSSION

Kunkel and Wallenius (10) found three electrophoretically distinct components in normal human hemolysate hemoglobin. In the present study, these same three components were found in zone electrophoretic separations of the hemoglobin associated with normal red cell membranes. However, the proportion of the minor basic component, hemoglobin A_2, which comprises 1.5 to 3.5 per cent of hemolysate hemoglobin (5), was

TABLE III

Per cent of electrophoretically slow (A_2) hemoglobin in water-soluble and membrane hemoglobin

Patients with thalassemia minor	Per cent hemoglobin A_2 in	
	Water-soluble hemoglobin*	Membrane hemoglobin†
D.M.	5.6	10.6
B.M.	4.7	11.9
O.N.	6.1	10.2
M.B.	5.0	10.9
Average	5.4	10.9

* Carbonmonoxyhemoglobin prepared by lysis of erythrocytes with 12 volumes of water.

† Carbonmonoxyhemoglobin prepared by homogenizing membranes in veronal buffer.

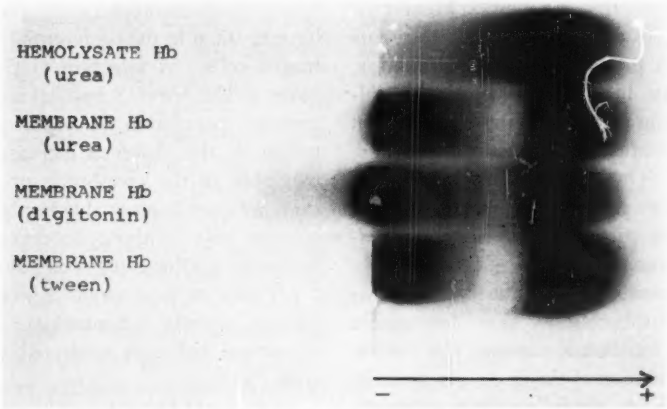

FIG. 2. STARCH GEL ELECTROPHORESIS OF HEMOLYSATE HEMOGLOBIN AND MEMBRANE HEMOGLOBIN PREPARED WITH VARIOUS AGENTS. Electrophoresis in borate buffer at pH 8.8 for 16 hours. Hemoglobin concentration of all samples 0.3 g per 100 ml; benzidine stain. Note the presence of A_2 component in all of the membrane preparations and apparent absence in the hemolysate at this concentration.

found to be increased (median 6.9 per cent) in the membrane pigment of normal subjects.

Comparative electrophoretic studies of membrane and hemolysate hemoglobin of individuals with uncomplicated sickle cell trait or hemoglobin C trait disclosed increased amounts of the abnormal hemoglobin (S or C) in the membrane preparations. The measurements of hemoglobin S or of hemoglobin C in both the hemolysate and the membrane preparations included hemoglobin A_2, but the amount of this minor component did not appear sufficient to account for the large increments of hemoglobin S or C observed in the membrane preparations. On three electrophoretic analyses of hemoglobin from individuals with sickle cell trait, sufficiently good separation of hemoglobin A_2 from hemoglobin S to allow separate quantification of these components was achieved. In these analyses, the average values for hemoglobin A_2 and hemoglobin S in the water-soluble pigment were 1.9 and 35 per cent, respectively; but in the membrane, hemoglobin A_2 accounted for as much as 5.3 per cent and hemoglobin S for no less than 68 per cent of the total pigment. Electrophoresis of the membrane hemoglobin of patients with sickle cell-hemoglobin C disease disclosed a higher proportion of hemoglobin C than of hemoglobin S in the membranes.

Appraisal of the validity of the methods employed in this study is necessary for the interpretation of the results. The reproducibility of analyses of chemical composition and hemoglobin content of red cell membranes prepared in this manner has been pointed out by Anderson and Turner (*2*); they also noted that concentrated solutions of urea did not change the spectrophotometric characteristics of cyanmethemoglobin. No new electrophoretic components which would suggest recombination of polypeptide chains (*11*, *12*) was encountered in analyses of the urea-treated hemoglobin, although the electrophoretic mobility of the S or C hemoglobin in membrane pigment was at times slightly slower than expected.

Increases in hemoglobin A_2, hemoglobin S and hemoglobin C in membrane preparations from normal individuals, those with sickle cell trait, and patients with sickle cell-hemoglobin C disease, respectively, were observed when urea was omitted and veronal was used to solubilize the membrane pigment. While some foaming was encountered in mechanically homogenizing the membranes in veronal buffer, similar treatment of water-soluble hemoglobin did not alter the expected distribution of electrophoretic components.

In the initial studies, membrane hemoglobin was solubilized in 50 per cent urea in Drabkin's diluent. Two other agents, 4×10^{-4} M digitonin in 0.015 M borate buffer, pH 8.9, and a solution of Tween 20 in normal saline, were also used to

solubilize the membrane pigment. Membrane hemoglobin prepared with any of the three agents contained a greater proportion of hemoglobin A_2 than did hemolysate hemoglobin (Figure 2), although differences in the amount of "trailing" on starch gel electrophoresis were evident in different preparations. The treatment of hemolysate hemoglobin with any of these agents did not appear to increase the proportion of electrophoretically slow minor component.

To study the possibility that the observed increase in the electrophoretically slow hemoglobin components in the membrane pigment was the result of preferential elution of the electrophoretically more rapid components, the following experiments were carried out. Standard membranes were prepared from a subject with sickle cell trait and from a subject with hemoglobin C trait. The first fraction of membrane pigment was then obtained by homogenizing the membranes in veronal buffer; the second and third fractions were obtained by successive additions to the centrifuged reddish stroma of 2 volumes of 50 per cent urea in Drabkin's diluent. The final residue after the second treatment with urea was a very small amount of reddish stroma. Starch gel electrophoresis of the three preparations of membrane pigment of both the S + A and the C + A erythrocytes revealed relative increases in the amounts of the electrophoretically slow components in all three fractions. The second and third fractions were too dilute for quantification on starch granules but no differences in the relative amounts of electrophoretically slow components of any of the three fractions were apparent on starch gel electrophoretic analyses.

Thus, membrane hemoglobin, prepared by several methods, contained relatively more of the basic components, A_2, S, or C, than did hemolysate hemoglobin. This increase seemed too regular and large to be an artifact.

Several possible explanations for these findings were suggested.

1) The possibility that the composition of membrane pigment reflected the selection of a certain erythrocyte population should be considered. Allison (13) found that the sickled osmotically resistant erythrocytes of individuals with sickle cell trait contained more sickle hemoglobin than did the osmotically fragile cells. The method of preparation of membranes in the present study might have resulted in the preferential loss of osmotically fragile cells. A selection of the cells containing more sickle (or C) hemoglobin from a heterogeneous population of erythrocytes might contribute to the observed increase in abnormal hemoglobin in the membrane preparations. However, we have been unable to prepare by fractional osmotic lysis (without deoxygenation) any populations of erythrocytes which differ by more than 5 per cent in hemoglobin S content, and no significant increase in hemoglobin A_2 has been noted in normal red cells subjected to graded osmotic lysis (5).

2) It might be postulated that membrane hemoglobin contains a hypothetical "membrane-hemoglobin A_1 complex" with the electrophoretic properties of hemoglobin A_2, S or C. The slight alterations in electrophoretic mobility of membrane A_2, S and C might well be related to the presence of non-hemoglobin constituents in the membrane preparations. Direct evidence concerning a "membrane-hemoglobin A_1 complex" is difficult to obtain. Rabbit hemoglobin contains no minor basic component, and no such component was demonstrable in rabbit membrane pigment. However, different species would be expected to have differences in chemical composition of erythrocyte membranes, and such a complex is a possible explanation of the findings of the present study.

3) At present the simplest explanation that can be offered for the observed differences in hemoglobin components is that A_2, S and C are indeed increased in relative amounts in the membranes when the membranes are prepared by the method specified. The fact that the more electropositive hemoglobin component was found to be increased in the membrane pigment suggests that the binding of hemoglobin to the membrane may be related to the hemoglobin charge. Some support for this interpretation may be derived from observations of the composition of membrane pigment in patients with sickle cell-hemoglobin C disease. While hemoglobin S accounted for 50 to 80 per cent of the membrane pigment of individuals with sickle cell trait (hemoglobin S + A), in the membrane hemoglobin prepared from S-C individuals, hemoglobin S accounted for only 28 per cent of the pigment. Ponder (14) summarized evidence that erythrocyte membranes contain strong acid

groups; the more electropositive hemoglobin components might thus be found in greater concentration in the membrane pigment.

Kaplan, Zuelzer and Neel (15) first observed that the principal morphologic abnormality associated with presence of hemoglobin C was the increased number of target cells on blood smears. The large amounts of hemoglobin C which were found in the erythrocyte membranes of individuals with sickle cell-hemoglobin C disease, or hemoglobin C trait may be related to the formation of target cells. A similar relationship between the sickling phenomenon and the presence of hemoglobin S in erythrocyte membranes was postulated by Anderson and Turner (1).

Target cells and microcytosis are frequently observed on blood smears of individuals with thalassemia minor. Electrophoretic analyses of the membrane hemoglobin of four individuals with thalassemia minor disclosed an increased amount of hemoglobin A_2 in the membrane pigment; however, the relative increase in hemoglobin A_2 in the red cell membranes of these individuals was not significantly different from the increases observed in normal erythrocyte membranes.

SUMMARY

1. Electrophoretic analyses of the hemoglobin associated with erythrocyte membranes disclosed the presence of the three known hemoglobin components (A_1, A_2 and A_3) in normal membranes. A larger proportion of hemoglobin A_2 was found in the membrane pigment than in water-soluble hemoglobin.

2. The membrane hemoglobin of individuals with sickle cell trait or hemoglobin C trait contained a larger proportion of the abnormal hemoglobin than did the water-soluble or hemolysate hemoglobin. The membrane hemoglobin of patients with sickle cell-hemoglobin C disease contained relatively larger amounts of hemoglobin C than did the water-soluble hemoglobin. While an increased proportion of hemoglobin A_2 was observed in the membrane pigment of individuals with thalassemia minor, the increase was within the range observed in membrane hemoglobin prepared from normal erythrocytes.

3. The results were consistent with the hypothesis that the binding of hemoglobin to the erythrocyte membrane is related to the hemoglobin charge, but other possible explanations for the observations were discussed.

ACKNOWLEDGMENT

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THE EFFECT OF CUSHING'S SYNDROME UPON SERUM ALBUMIN METABOLISM *

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The present investigation of albumin metabolism in Cushing's syndrome employing the I^{131}-labeled albumin turnover method (1) was undertaken in view of clinical and experimental evidence of significant effects of steroid hormones upon protein metabolism. Previous reports have described acute effects of large doses of glucocorticoids in human subjects (2–4). The effects of chronic hyperadrenalism were examined in the present work.

MATERIAL AND METHODS

Clinical material

The following four categories of subjects were studied: *1*) normal volunteers, *2*) physically healthy hospitalized schizophrenic patients, *3*) cases of Cushing's syndrome due to adrenal cortical hyperplasia; and *4*) a patient with Cushing's syndrome due to exogenous steroids.

1) *Normal volunteers.* Thirteen subjects, 10 male, 3 female with ages ranging from 19 to 40 years.

2) *Physically healthy hospitalized schizophrenic patients.* Ten subjects, 5 male, 5 female with ages ranging from 17 to 39 years.

3) *Cushing's syndrome.* Albumin turnover studies were carried out on 4 women during the active stage of clinically typical Cushing's syndrome attributed to bilateral adrenal hyperplasia. Three of the 4 were studied again after therapy and remission. The diagnoses were supported by elevated urinary ketogenic steroids or corticoids (Porter-Silber chromogens). In M.W., administration of prednisone was followed by suppression of urinary 17-ketosteroid levels. In the other 3 subjects, intravenous ACTH was followed by an exaggerated rise in plasma corticoids, considered indicative of hyperplasia rather than tumor (5). None of the women exhibited appreciable virilization. All had amenorrhea; the 3 of pre-menopausal age resumed menstruation after therapy.

M.W., aged 36, had severe Cushing's syndrome with osteoporosis and collapsed lumbar vertebrae, hypertension, intermittent hypokalemic alkalosis, and increased requirement of insulin for diabetes mellitus which apparently antedated the onset of hyperadrenalism. After complete removal of enlarged adrenal glands, the patient resumed menstruation for the first time in several years. She was given relatively large steroid doses in an effort to minimize orthostatic hypotension and episodes of Addisonian crisis associated with intercurrent infections. When restudied with I^{131}-albumin 15 months after total adrenalectomy, the patient was receiving 30 mg cortisol, 0.5 mg 9-α-fluorocortisol, and 1 g tolbutamide daily.

C.H., aged 50, had Cushing's syndrome of moderate severity with hypertension but no diabetes or osteoporosis. She had facial rounding, cervico-dorsal and supraclavicular fat pads, and thin skin with a few pink striae. She had been emotionally disturbed and required care in a mental hospital during both initial and follow-up studies. She was restudied with I^{131}-albumin 10 months after total adrenalectomy, while receiving 20 mg cortisone daily.

R.H., aged 32, had Cushing's syndrome of minimal severity with hypertension but no impaired glucose tolerance or osteoporosis. She had some facial rounding, hirsutism, amenorrhea, easy bruisability, and increased pigmentation. Following irradiation of the pituitary (4,080 r), the elevated urinary 17-ketosteroids and ketogenic steroids fell to the normal range. Menses reappeared for the first time in 7 months. The patient's general appearance was improved at the time of the second I^{131}-albumin study 10 months after radiotherapy.

A.G., aged 38, had mild Cushing's syndrome with amenorrhea, facial rounding, acne, hirsutism, supraclavicular fat pads, and ecchymoses. After removal of one hyperplastic adrenal gland, she underwent what was considered coincidental partial remission with return of menses. During the next 6 years there were intermittent manifestations of moderate activity of the disease. This was followed by a severe re-exacerbation with hypertension, glycosuria, and osteoporosis with a collapsed lumbar vertebra. During the I^{131}-albumin turnover study, the patient received radiotherapy to the pituitary without diminution of the elevated urinary ketogenic steroid values. Four months after the pituitary irradiation (approximately 4,000 r), there was general clinical improvement and recurrence of menses. The elevated urinary ketogenic steroids fell to normal. A follow-up I^{131}-albumin study was not carried out.

4) *Cushing's syndrome due to exogenous steroids.* E.B., a 43 year old woman, had dermatomyositis verified

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by biopsy. The initial I^{131}-albumin study was done during the third month of cortisone therapy (200 mg per day). A later albumin study was carried out during the second month of a higher dosage schedule (300 mg per day). During both studies, the patient presented the typical appearance of Cushing's syndrome with marked facial rounding and "buffalo" obesity.

Methods

Total serum protein concentrations were determined in duplicate by the biuret method, employing two or more known standards prepared by dry weight or checked by Kjeldahl analysis. The albumin values were obtained by paper electrophoresis in duplicate with the standard Spinco apparatus and Analytrol. The albumin concentration was determined at least twice in each subject, at the beginning and end of the turnover study, with additional intermediate determinations in many instances. The albumin values obtained in each subject agreed within 0.4 g per 100 ml or less.

The radioiodinated serum albumin shipments were obtained from E. R. Squibb and Sons or from Abbott Laboratories. Immediately upon receipt of each lot from Abbott Laboratories, carrier albumin (25 to 50 mg Red Cross albumin) was added to the vial to minimize radiation damage to the labeled protein (6). The Squibb solutions were stated to contain about 10 to 15 mg protein per ml, which was considered satisfactory from the standpoint of self-irradiation.

Each hyperadrenal patient was studied simultaneously with two or more control subjects to avoid any possible discrepancies that could arise from different behavior *in vivo* of individual shipments of radioiodinated albumin. No variation between different lots was observed. Urine collections during the first day after injection revealed that amounts not exceeding 10 per cent of the injected radioactivity had been excreted, a finding compatible with absence of appreciable amounts of abnormal rapidly degraded components (1, 7).

After 1 day or more of iodine prefeeding (Lugol's solution, 15 drops daily), the subjects received 50 to 100 μc of I^{131}-labeled albumin by intravenous injection. Heparinized venous plasma samples were obtained 10 minutes after injection, and daily or three times weekly for three or more weeks.

In several of the experimental and control subjects, 24-hour urine collections were obtained during most or all of the study period. The completeness of the collections was verified by creatinine determinations.

The plasma samples of each subject were counted with appropriate standards in a well-type scintillation counter, usually at the conclusion of the study, thus obviating the need for physical decay corrections. Other samples, including urines, were corrected when necessary for physical decay.

The data on plasma radioactivity were treated as originally described (1). Disappearance curves were obtained from semilogarithmic plots of the plasma radioactivity against time (Figures 2 and 3). The rapid initial fall of the first few days, attributed to distribution of the tracer throughout the "total exchangeable albumin pool," was followed by a gradual linear decline, the exponential decay due to metabolic degradation. Lines were drawn by inspection, or by the method of least squares when the points showed appreciable scatter. The half-time ($T_{\frac{1}{2}}$) of the slow component was obtained by graphic analysis. From the $T_{\frac{1}{2}}$ the equation, $0.693/T_{\frac{1}{2}} = k$, gave the fractional turnover rate, k, expressed as per cent of the albumin pool replaced per day. The total exchangeable albumin pool in grams was obtained by the isotope dilution formula, employing the quotient:

$$\frac{\text{total radioactivity injected}}{\text{radioactivity per gram of albumin}}.$$

The denominator was obtained from the zero time extrapolation of the slow component which represented plasma radioactivity if distribution had occurred instantaneously upon injection. The product of the pool and turnover rate yielded the degradation rate in grams per day. The rate of synthesis should be the same as the degradation rate under steady state conditions, which were assumed to exist in the absence of significant clinical alterations. The observed constancy of the serum albumin concentration was compatible with this assumption. The data for the albumin pools and degradation rates were adjusted to 1.73 m^2 surface area. The intravascular albumin (circulating plasma albumin) in grams was calculated from the 10 minute value for radioactivity per gram of albumin used as the denominator in the isotope dilution formula.

The data on urinary radioactivity were treated by two types of computation described by Rothschild, Schreiber, Oratz and McGee (2) and by Berson, Bauman and Rothschild (7–9). One value for the albumin degradation rate was obtained from the quotient:

$$\frac{\text{per cent renal excretion per day}}{\text{per cent injected radioactivity retained in the body}},$$

which was multiplied by the total exchangeable albumin pool (7, 8). A second value was the product of the "metabolic clearance" of I^{131}-albumin and the mean serum albumin concentration (2, 9). The results of both methods based upon urinary data proved to be in reasonable agreement with the values obtained from plasma disappearance curves, which were used in Table I and in Figures 1, 2 and 3. Extensive discussions of the validity and limitations of I^{131}-albumin turnover studies have appeared previously (6, 7, 10–13).

RESULTS AND INTERPRETATIONS

The normal control and schizophrenic groups had biological half-times of albumin turnover (mean $\pm$ standard deviation) of 13.4 $\pm$ 1.3, and 13.5 $\pm$ 1.3 days, respectively (Figure 1 and Table I). No significant variation related to age or sex was observed.

TABLE I

Albumin turnover data in Cushing's syndrome and control groups

Condition	Subject	Sex	Age	Wt	Surface area	Status	Serum albumin	Kinetic and isotope dilution data: Kinetics of disappearance $T_{\frac{1}{2}}$	Kinetics of disappearance Turnover rate	Total exchangeable albumin pool		Degradation rate	
			yrs	*kg*	m^2		g %	*days*	%/*day*	*g*	*g/1.73 m^2*	*g/day*	*g/day/1.73 m^2*
Cushing's syndrome	M.W.	F	36	66.0	1.65	Disease active; osteoporosis	3.8	7.7	9.0	170	178	15.3	16.0
			37	61.9	1.60	15 mos. after adrenalectomy	2.7	10.8	6.4	208	225	13.3	14.5
Cushing's syndrome	C.H.	F	50	65.0	1.62	Disease active;	4.7	9.7	7.2	250	267	17.9	19.1
			51	46.8	1.41	10 mos. after adrenalectomy	4.3	14.3	4.8	266	326	12.9	15.8
Cushing's syndrome	R.H.	F	32	63.6	1.66	Disease active;	4.6	11.4	6.1	293	305	17.9	18.7
			33	63.2	1.66	10 mos. after pit. irradiation	4.4	15.4	4.5	394	411	17.7	18.5
Cushing's syndrome	A.G.	F	38	56.6	1.58	Disease active; osteoporosis	3.6	8.5	8.2	179	196	14.6	16.0
						Active Cushing's syndrome: Mean		9.3	7.6	223	237	16.4	17.5
						SD		1.6	1.2	59	60	1.7	1.7
						SEM*		0.8	0.6	29	30	0.9	0.8
Cortisone therapy	E.B.	F	43	75.0	1.78	3rd mo., 200 mg cortisone daily;	4.2	10.1	6.9	261	254	18.0	17.5
			43	75.0	1.78	2nd mo., 300 mg cortisone daily	4.7	8.6	8.1	261	254	21.0	20.4
Normal						13 Subjects: Mean		13.4	5.2	314	300	16.3	15.6
						SD		1.3	0.5	48	37	2.9	2.2
						SEM		0.4	0.1	13	10	0.8	0.6
Schizophrenia						10 Subjects: Mean		13.5	5.2	323	318	16.6	16.4
						SD		1.3	0.5	64	37	3.0	1.8
						SEM		0.4	0.2	20	12	1.0	0.6

* Standard error of the mean.

The subjects with active Cushing's syndrome had significantly shorter half-times with a mean value of 9.3 ± 1.6 days ($p < 0.01$). This acceleration of albumin turnover was reversed by treatment of the disease. As seen in Figures 1, 2 and 3 and in Table I, the $T_{\frac{1}{2}}$ became appreciably longer, returning to or toward the normal range in the three subjects restudied during remission following therapy. Repeated studies in control subjects at intervals as long as 1 year have revealed no detectable change (14).

The mean value for the total exchangeable albumin pool was significantly reduced in the cases of Cushing's syndrome as compared with the normal ($p < 0.05$) and schizophrenic groups ($p < 0.01$). The albumin pools were smallest in M.W. and A.G., the two subjects with the shortest half-times and with the most severe clinical manifestations, which included advanced osteoporosis. Nevertheless, there was no appreciable hypoalbuminemia.

Subject E.B., who was first studied while receiving 200 mg, and again while receiving 300 mg cortisone daily for dermatomyositis, also showed accelerated albumin turnover, more pronounced on the higher dose (Figure 1 and Table I). It should be mentioned that dermatomyositis per se was not found to be associated with abnormal albumin metabolism (14).

The total exchangeable albumin pool did not change in E.B. on the two different doses of cortisone. In contrast, each of the cases of Cushing's syndrome had a higher value for the albumin pool when restudied after therapy. With the increased albumin pool, M.W. exhibited an unchanged intravascular fraction of the pool (34 per cent, first study; 33 per cent, second study). The serum albumin concentration was diminished in association with expanded plasma volume. The precise cause of this hypervolemic hypoalbuminemia, and the possible contribution of excess salt and water retention with high steroid dosage were not clear. The other cases, C.H. and R.H., showed slight diminutions of the serum albumin concentration,

opposite in direction to the increased values for the albumin pools.

No consistent variations in intravascular-extravascular distribution were observed. The mean values ± standard deviations for the intravascular per cent of the total exchangeable albumin pool were: normal control group, 40.2 ± 4.2 per cent; schizophrenic group, 41.2 ± 7.2 per cent; and active Cushing's syndrome, 38.5 ± 5.4 per cent.

It was not unexpected that the degradation rates in Cushing's syndrome, being the products of diminished pools and increased turnover rates, revealed no marked deviations from the normal range. The values for albumin degraded and synthesized daily revealed some diminution after therapy in M.W. and C.H., but no appreciable change in R.H. Subject E.B. showed an increased degradation rate on the larger as compared with the smaller dose of cortisone.

DISCUSSION

On theoretical grounds it has been shown (12) that the methodology employed probably results in an overestimation of the total exchangeable albumin pool and that, in general, this overestimation becomes more serious with shorter biological half-times (12, 13). In the present study, the *lower* values for albumin pool in active Cushing's syndrome cannot be attributed to computational error, since the associated short half-times would tend to exaggerate the overestimation of total exchangeable albumin.

The findings illustrated accelerated albumin turnover associated with the excess glucocorticoids of spontaneous or exogenously induced Cushing's syndrome. This abnormality was reversed by definitive therapy and was accentuated by increased steroid dosage. The extent of deviation from normal appeared to be related to the severity of the clinical manifestations.

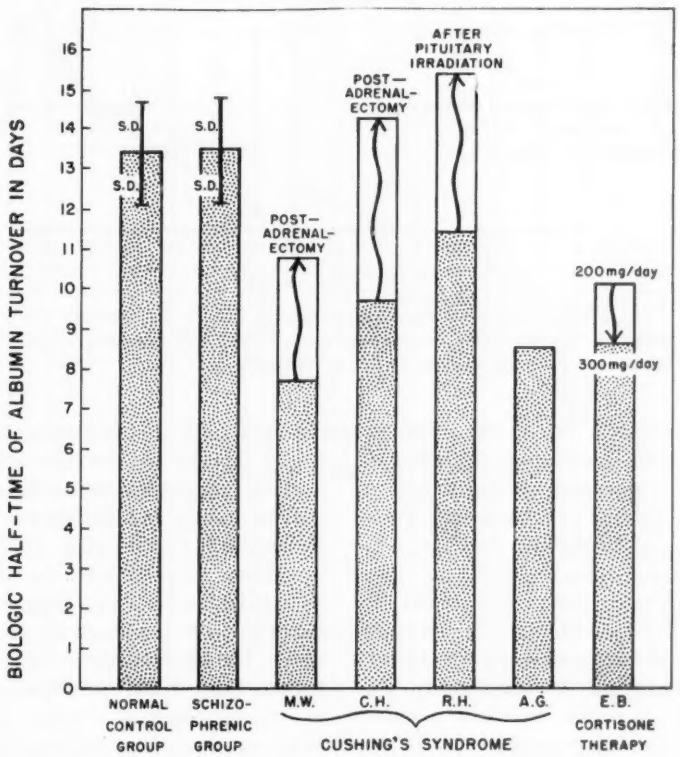

FIG. 1. EFFECT OF CUSHING'S SYNDROME UPON SERUM ALBUMIN TURNOVER.

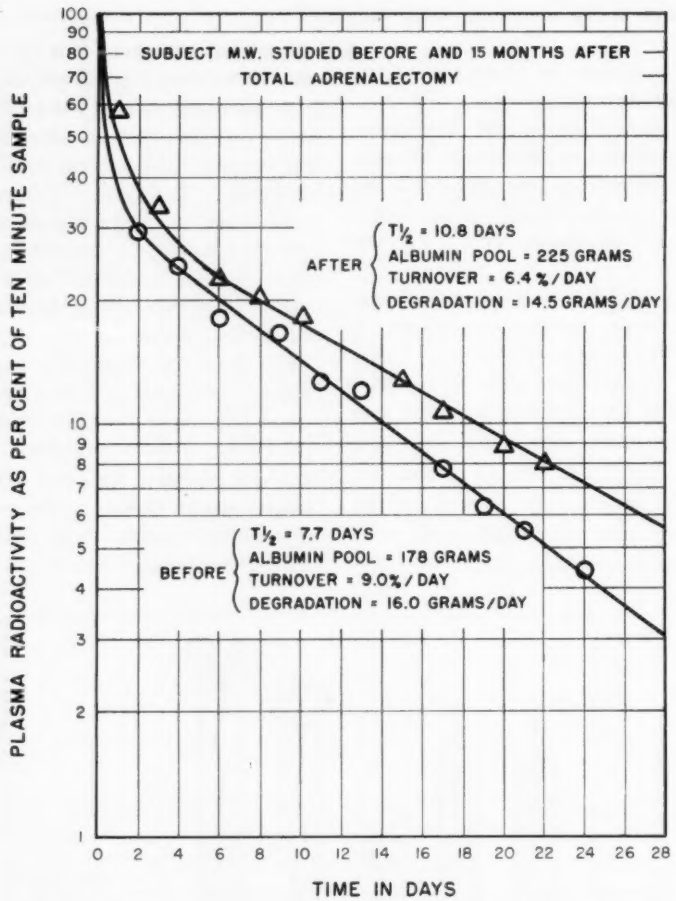

FIG. 2. I^{131}-LABELED ALBUMIN DISAPPEARANCE CURVE IN CUSHING'S SYNDROME IN SUBJECT M.W. (SEMILOGARITHMIC PLOT). The albumin pools and degradation rates are adjusted to 1.73 m^2 surface area.

Human subjects started on large doses of glucocorticoids have manifested acutely increased albumin degradation, according to the reports of Rothschild and co-workers (*2*), Grossman, Yalow and Weston (3), and Blythe and associates (4).

Accelerated albumin turnover is by no means confined to Cushing's syndrome. It is seen in thyrotoxicosis (11, 14) as well as in the hypoproteinemias associated with proteinuria (8, 13, 14) and with gastrointestinal protein-wasting syndromes (15–18). It is not, however, regularly associated with either malnutrition (14, 19) or debilitating illness. Subjects with marked wasting, including patients with advanced carcinoma, myotonic muscular dystrophy, and miscellaneous muscle diseases did not have accelerated albumin turnover, although some subjects had marked hypoalbuminemia and diminution of the total exchangeable albumin pool (14). This finding would tend to exclude the possibility that the accelerated turnover in Cushing's syndrome might be attributable to nonspecific effects of severe illness. The close similarity of the findings in the normal control and schizophrenic groups signifies the constancy of albumin metabolism despite emotional illness and wide variations in behavior.

The mechanism of the accelerated albumin turnover in Cushing's syndrome cannot be stated ex-

plicitly at present. Certain inferences are warranted, however. In the absence of significant clinical alterations and with constancy of the serum albumin concentration, the subjects were considered to be in a "steady state" during the several weeks of each study. Therefore, the rate of synthesis was presumed to be equal to the rate of degradation. After therapy and remission, the computed degradation rate fell in two of the three cases; the albumin pool rose in each. Since the total exchangeable albumin values were not markedly diminished in active Cushing's syndrome (Table I), it may be inferred that accelerated albumin catabolism was at least partly compensated for by increased synthesis of albumin.

Conceivably, small discrepancies between synthesis and degradation persisting over long periods (months or years) may have led to the observed diminution in albumin pool. However, since Cushing's syndrome had been present for some time, and since the follow-up studies were done 10 to 15 months after therapy, no definite statement can be made concerning the precise sequence of the changes. As noted previously, the smallest albumin pools were found in the subjects with the shortest half-times, M.W. and A. G., with the most severe clinical manifestations, which included advanced osteoporosis.

An instance of compensatory increase in albumin synthesis is seen in Subject E.B. On the

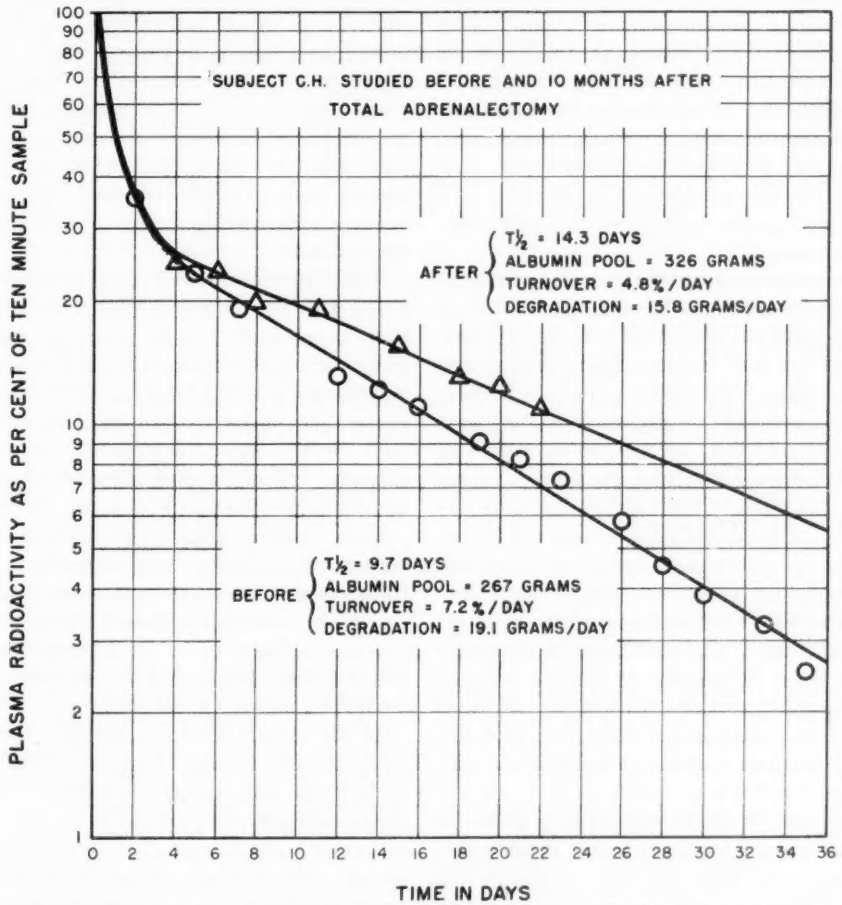

FIG. 3. I^{131}-LABELED ALBUMIN DISAPPEARANCE CURVE IN CUSHING'S SYNDROME IN SUBJECT C.H. (SEMILOGARITHMIC PLOT). The albumin pools and degradation rates are adjusted to 1.73 m^2 surface area.

higher steroid dose there was an accelerated turnover with increased rate of degradation and presumably of synthesis, but the value for total exchangeable albumin remained unchanged.

Further evidence bearing on this point is available in the report by Rothschild and co-workers (*2*) which describes the acute effects of 13 to 19 days of cortisol or prednisone administration. By injection of a second I^{131}-albumin tracer during the period of steroid medication, it was possible to calculate a second total exchangeable albumin pool. The diminution in albumin pool associated with hormone treatment was definitely less than that expected from the increased degradation rate. Thus, in four male subjects, the authors reported a mean figure of 59 g as the increase in albumin degraded during steroid administration. Since the mean diminution in albumin pool was only 31 g, the difference, 28 g, was attributed to increased albumin synthesis. Even allowing for errors implicit in the methodology, it would appear likely from these data and from the present report, that glucocorticoids increase albumin synthesis as well as degradation under both short-term and long-term conditions.

The promotion of increased gluconeogenesis by adrenal cortical hormones has been the subject of much work since the animal experiments of Long, Katzin, and Fry (*20*) and the clinical observations of Albright (*21*). The detailed study by Hoberman (*22*) employed N^{15}-glycine in fasting rats treated with cortisone, as well as in rats with increased endogenous steroid production in response to cold stress. The findings indicated that excess glucocorticoids caused acceleration of the catabolism of body protein to amino acids, as well as the catabolism of amino acids to urea, but caused reduction of the synthesis of body protein from amino acids. The patients with Cushing's syndrome given N^{15}-glycine by Parson, Crispell and Ebbert (*23*) revealed a markedly increased rate of N^{15} excretion in the urine, despite maintenance of nitrogen equilibrium as shown by balance study.

The evidence of the protein-wasting action of the adrenal cortical hormones refers to the overall effect upon the body, but does not require the assumption that the same processes occur in the different tissues. Direct evidence to the contrary is provided by animal experiments showing net protein anabolism in some tissues simultaneously with catabolism in others. Silber and Porter observed wastage of nitrogen from the carcasses of cortisone-treated rats, with concurrent increase in the total protein of the liver and other viscera as well as increased plasma albumin concentration (*24*). Clark carried out analyses of the carcasses and livers of control and cortisone-treated rats which were given N^{15}-glycine (*25*). The lower N^{15} content in carcass protein of the cortisone-treated animals indicated diminished protein synthesis. In contrast, the liver protein nitrogen and N^{15} were increased in the treated rats, as were the serum protein concentration and its N^{15} content. The increased urinary nitrogen and N^{15} excretion observed was attributed to the reduced protein synthesis in the carcass which outweighed the increased hepatic activity in serum and liver protein synthesis. The increased metabolism of the liver was viewed as probably a "compensatory" reaction. Such may also be the case in the present study in which increased hepatic albumin synthesis presumably tended to offset increased degradation.

This difference in the behavior of liver as compared with muscle and carcass protein is not entirely unexpected. Earlier work such as the N^{15} glycine studies of Shemin and Rittenberg (*26*), and Sprinson and Rittenberg (*27*) revealed the greater protein turnover of the liver as compared with the less metabolically active tissues of the carcass. It would appear, then, that hepatic synthetic mechanisms either have the ability to compensate, or perhaps are directly stimulated by experimental or clinical hyperadrenalism. In contrast, impaired ability to compensate for increased catabolism in less active tissues would produce the protein-wasting seen in the osteoporosis, muscle atrophy, thin skin, and diminished fibrous tissue repair of Cushing's syndrome.

In the present group of patients who showed no appreciable virilization, the abnormalities were ascribed to the demonstrated excess of 17-hydroxycorticosteroids, which were not subjected to further analysis, such as chromatographic separation. The findings in virilizing adrenal disorders and the effects of the adrenal androgen, dehydroisoandrosterone (*28*), remain to be explored.

SUMMARY

1. I^{131}-labeled albumin turnover studies were carried out in four patients with Cushing's syndrome due to adrenal cortical hyperplasia and in control subjects.

2. In active Cushing's syndrome there was a short biological half-time of the tracer, signifying accelerated turnover.

3. This abnormality was reversed after therapy and remission.

4. Cushing's syndrome due to the administration of cortisone also revealed accelerated albumin turnover, more pronounced with a larger dose.

5. The total exchangeable albumin pool was reduced in Cushing's syndrome, and was smallest in the two subjects with the shortest half-times and with the most severe clinical manifestations.

6. Excess glucocorticoids were considered to have produced accelerated albumin catabolism, partly compensated for by increased synthesis.

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RENAL MECHANISMS FOR THE EXCRETION OF INORGANIC SULFATE IN MAN *

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The sulfur contained in plasma is derived from sulfalipids, sulfatides, amino acids, and glycoproteins as well as from ionizable inorganic sulfate. The plasma concentration of inorganic sulfate is partially determined by the kidneys, since the ion is freely filterable at the glomerulus (1, 2) and reabsorbed by the renal tubules. In dogs (3) the excretory processes have been fairly well defined and the tubular reabsorptive mechanism has been shown to be characterized by a maximal transfer rate (Tm_{SO_4}). The present study was undertaken to ascertain if similar mechanisms are operating in man. To elucidate this problem, the filtered load, tubular reabsorption, and urinary excretion of inorganic sulfate were measured prior to and during the infusion of sodium sulfate solution. Further, the readily exchangeable sulfate mass was determined using isotope dilution techniques. The results of this study show that the tubular reabsorption of inorganic sulfate in man is limited by a maximal transfer rate (Tm).

MATERIALS AND METHODS

Subjects. The subjects were convalescent patients without apparent cardiovascular, renal, or metabolic disorders, and healthy young adult volunteers. All subjects had been on an ordinary diet and were fasted 12 hours prior to the study.

Plan of study. Control blood and urine samples were used to determine the fasting plasma inorganic sulfate concentration and the inulinoid blank in urine and plasma. To measure the glomerular filtration rate (GFR) a priming dose of inulin, administered to raise the blood level to approximately 0.25 mg per ml, was followed by a sustaining infusion containing inulin in saline, delivered by a Bowman constant infusion pump at a rate of 1.0 ml per minute.

After an appropriate equilibration time, at least two urine collection periods were obtained for measurement of control glomerular filtration and sulfate excretion. Five per cent sodium sulfate [1] was subsequently administered by a second constant infusion pump to insure an independent rate of infusion of 2 to 10 ml per minute. Throughout each study, which lasted for about 4 hours, blood samples were obtained and urine collections were made at intervals of 30 to 40 minutes. Complete urine collections were obtained by urethral catheterization and bladder washout with 20 ml of sterile distilled water followed by an equal volume of air.

Analytical methods. Inulin was determined by Schreiner's (4) modification of the method of Roe, and inorganic sulfate was analyzed by the method of Power and Wakefield (5).

Determination of the exchangeable sulfate mass. The readily available inorganic sulfate mass was calculated as the product of the fasting plasma inorganic concentration (P_{SO_4}) and the simultaneously measured volume of distribution of radiosulfate ($V_{S^{35}O_4}$). The volume of distribution of radiosulfate was derived by dividing the total administered radioactivity by the plasma radioactivity at the time of injection (6). This latter value was obtained by extrapolating the exponential disappearance curve of the injected radiosulfate to zero time. No correction was made for urinary loss of radiosulfate during the equilibration time. According to Ryan, Pascal, Inoye and Bernstein (7), as much as 7.2 per cent of the administered dose is lost during equilibration. Therefore, the plasma concentration of radiosulfate obtained in this study is too high and the calculated volume of distribution is smaller than the extracellular fluid. However, the data obtained suffice for the needs of this study. The dried samples of plasma and urine, corrected for self-absorption, were counted with a windowless gas-flow counter.

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[1] Sodium sulfate for intravenous administration was kindly supplied by Abbott Laboratories of North Chicago, Ill.

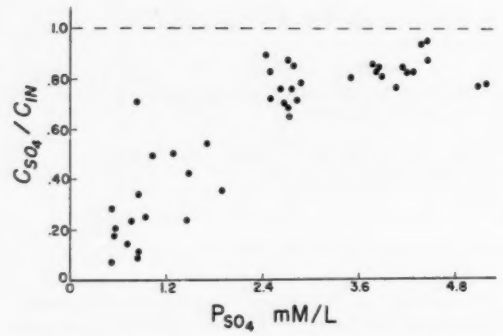

FIG. 1. THE PLASMA SULFATE CONCENTRATION PLOTTED AGAINST THE CLEARANCE RATIOS OF SULFATE AND INULIN.

Calculations. The tubular reabsorption of inorganic sulfate (T_{SO_4}) was calculated as:

$$T_{SO_4} = [(P_{SO_4})(GFR)] - (U_{SO_4}V) \qquad [1]$$

where P_{SO_4} and U_{SO_4} represent the plasma and urine concentrations of inorganic sulfate in micromoles per milliliter; GFR, glomerular filtration rate in milliliters per minute; and V, the urine flow in milliliters per minute. The maximal tubular reabsorption of sulfate (Tm_{SO_4}) was calculated by averaging the three highest values for tubular reabsorption during elevated plasma inorganic sulfate concentrations. This calculation was not corrected for equilibration of sulfate across a semipermeable membrane. Swan, Feinstein and Madisso (2) reported an equilibrium constant of 0.95 for man. If this factor had been applied, the load and Tm would have been slightly smaller.

RESULTS

Fasting plasma sulfate concentration. The fasting plasma sulfate concentration in millimoles of inorganic sulfate per liter of plasma, determined in 31 patients, varied between 0.2 and 1.1 mmoles per L (mean 0.6 ± 0.2 mmole per L). No relationship was noted between the plasma sulfate concentration and age, sex, body weight, or readily exchangeable sulfate mass.

Fasting sulfate clearance. The fasting sulfate clearance ($U_{SO_4}V/P_{SO_4}$) in 12 subjects ranged from 7.1 to 61.3 ml per minute. In the fasting

TABLE I

Data obtained in three studies of a representative subject

GFR	Plasma sulfate	Sulfate			$(P_{SO_4} \times GFR)/T$	(T/GFR)100	T/BW
		Filtered	Excreted	Reabsorbed			
ml/min	*mmoles/L*		*μmoles/min*			*μmoles/min/100 ml GF**	*μmoles/min/kg*
No. 1 control							
98.0	0.55	54.0	5.7	48.3	1.1	49.3	0.8
98.0	0.55	54.0	5.8	48.2	1.1	49.3	0.8
Sulfate infusion							
98.0	1.43	140.0	3.22	107.8	1.3	110.3	1.8
114.0	1.52	173.0	73.0	100.0	1.7	88.0	1.7
114.5	1.95	223.0	130.0	93.0	2.4	81.3	1.6
No. 2 control							
99.1	0.61	60.5	2.9	57.6	1.1	58.0	1.0
81.7	0.52	42.5	2.0	40.5	1.1	49.5	0.7
Sulfate infusion							
86.9	1.08	93.8	39.8	54.0	1.7	62.2	0.9
105.8	1.21	128.0	58.2	69.8	1.8	66.0	1.2
101.9	1.30	132.5	80.6	51.9	2.6	51.0	0.9
89.4	1.70	152.0	95.0	57.0	2.7	63.8	1.0
No. 3 control							
83.4	0.39	32.5	4.7	27.8	1.2	33.3	0.5
83.4	0.39	32.5	5.8	26.7	1.2	32.0	0.5
Sulfate infusion							
80.4	0.60	48.2	7.2	41.0	1.2	51.0	0.7
77.5	0.84	65.1	32.1	33.0	2.0	42.7	0.6
87.3	1.23	107.4	65.2	42.2	2.6	48.5	0.7

* Micromoles per minute per 100 ml of glomerular filtrate (GF).

TABLE II

Average data of all subjects

Patient	BW	GFR	T (control)	Tm	$(P_{SO_4} \times GFR)/Tm$*	T/Tm	(Tm/GFR) ×100	Tm/BW
	kg	*ml/min*	*μmoles/min*	*μmoles/min*		*%*	*μmoles/min/100 ml GF*†	*μmoles/min/kg*
CA no. 1	62.2	88	47	81	2.3	58.0	92.0	1.3
no. 2	62.2	109	65	84	3.6	77.4	77.1	1.4
JO no. 1	59.1	87	98	189	2.4	51.9	217.2	3.2
no. 2	59.1	87	124	143	2.1	86.7	164.3	2.4
NO no. 1	70.9	125	56	149	2.4	37.6	119.2	2.1
no. 2	70.9	156	61	111	4.7	55.0	71.2	1.6
AD no. 1	65.5	130	108	150	2.3	72.0	115.4	2.3
no. 2	65.5	147	71	188	6.8	37.8	127.8	2.9
WE no. 1	74.5	128	81	154	3.5	52.6	120.3	2.1
	74.5	118	75	168	3.8	44.6	142.4	2.3
HE	65.5	89	34	36	2.2	94.4	40.4	0.6
RO	59.0	100	33	47	4.9	70.2	47.0	0.8
PA	63.6	125	66	156	1.7	42.3	124.8	2.5
MI	62.7	112	47	75	3.2	62.7	66.9	1.2
DJ	60.9	98	32	47	4.8	68.1	47.9	0.8
GA	63.6	88	15	50	5.0	30.0	56.8	0.8
CL	81.8	98	47	53	4.8	88.6	54.1	0.7
EF	69.8	144	77	166	3.1	46.4	115.3	2.4
ST	73.2	175	91	172	3.6	52.9	98.3	2.4
RI	43.2	84	17	34	5.9	50.0	40.5	0.8

* Highest load/Tm ratio; Tm = average of 3 periods with elevated sulfate load.
† Micromoles per minute per 100 ml of glomerular filtrate (GF).

state tubular reabsorption of inorganic sulfate is close to maximal. Therefore, as the filtering load $[(GFR)(P_{SO_4})]$ is increased, the clearance of sulfate also increases, approaching GFR, less the amount of sulfate reabsorbed by the tubules (Figure 1).

Tubular reabsorption of inorganic sulfate. In 23 studies on 16 subjects, tubular reabsorption of inorganic sulfate was determined before and during the administration of sodium sulfate. In all instances a maximal rate of sulfate reabsorption (Tm_{SO_4}) was reached, and this was not increased by further elevation of the plasma sulfate concentration. The data obtained in three studies of a representative patient are shown in Table I. There was no reproducibility of fasting inorganic sulfate concentration on successive weekly studies. Variations are not due to changes in filtration rate, since this was remarkably constant. The load/T ratios in this patient ranged from control level of 1.05 to a high of 2.67 during sulfate infusion, well above that considered necessary to saturate the transfer mechanism. At these load/T ratios, no evidence of the self-depression phenomenon was noted.

Data for all other patients are summarized in Table II. Tubular reabsorption of inorganic sulfate varied markedly among individual patients and in repeated determinations in the same subject. The mean Tm_{SO_4} was 113 mmoles per minute (SD ± 55) and the coefficient of variation was

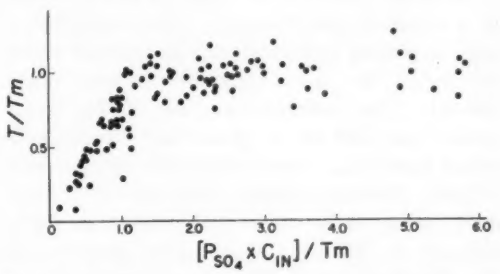

FIG. 2. INORGANIC SULFATE T/TM PLOTTED AGAINST THE SULFATE LOAD/TM RATIOS.

49 per cent. If the Tm_{SO_4} is related to 100 ml of glomerular filtrate, the mean was 97 mmoles per minute (SD ± 45) with a coefficient of variation of 47 per cent. Since there is no reproducibility and since the variation within each study is too great to permit comparison of the Tm_{SO_4} among individuals, both the T_{SO_4} and the filtered load ($P_{SO_4} \times GFR$) were factored by the maximal tubular transfer rate (Tm_{SO_4}). These values, for all subjects, are plotted in Figure 2. Accepting the existence of a Tm, this figure illustrates that the T/Tm ratio approaches 1.0 as the load/Tm is raised above 1.0, thereafter remaining constant to load/Tm ratios of 5.0.

DISCUSSION

The observed fasting plasma inorganic sulfate concentration of 0.6 mmole per L (equivalent to 1.9 mg per 100 ml sulfur, or 1.2 mEq per L sulfate) is in agreement with values summarized by Hayman in 1932 (8) and later confirmed by others (5, 7, 9–11). The plasma inorganic sulfate level is determined by many variables, such as protein catabolism, absorption from the gastrointestinal tract, and glomerular-tubular balance. As far as we know, the only variable that can be directly controlled is the dietary protein. The values obtained in this study represent an unselected sampling, since no attempt was made to regulate the diet.

With increasing loads, the sulfate clearance will approach glomerular filtration asymptotically because tubular reabsorption of sulfate is rapidly exceeded, and therefore the amount of filtered sulfate becomes relatively much greater than the amount of sulfate reabsorbed by the tubules.

The results of this study show that reabsorption of inorganic sulfate by the renal tubules is limited by a maximal rate (Tm_{SO_4}). The variability of this Tm among individuals and in repeated observations in the same individual remains unexplained. This variability persists, if Tm is expressed per 100 ml of glomerular filtrate, indicating that Tm_{SO_4} is not comparable between individuals. Further, tubular reabsorption of inorganic sulfate is in man not dependent on GFR, although in the dog, Tm_{SO_4} varies directly with the filtration rate (12). In determination of Tm_{SO_4}, it cannot be excluded that there is diffusion of sulfate from peritubular fluid to tubular urine as shown in the dog after a single injection of radiosulfate ($S^{35}O_4$) into the renal artery (13). To explore mechanisms which might conceivably be interdependent and affect the tubular reabsorption of sulfate, the readily available sulfate mass was related to the Tm_{SO_4} but no correlation was noted.

Since sulfate is apparently actively reabsorbed in the kidney by a transfer mechanism showing limited capacity, it should follow the principle of an enzyme catalyzed reaction with a maximal reaction rate (Tm). Shannon in 1939 postulated (14) the general processes for the excretion of substances characterized by a Tm. Thompson and Hiatt (15) applied this concept to inorganic phosphate. The hypothesis could similarly be utilized for inorganic sulfate reabsorption.

Cohen, Berglund and Lotspeich (16) have reported that acetoacetate, phosphate, and sulfate share a common reaction with glucose during tubular reabsorption. They further demonstrated that glucose was preferentially reabsorbed, a reaction selectively inhibited by phlorizin, accelerating the reabsorption of acetoacetate, phosphate and sulfate.

Berglund and Lotspeich (12, 17) found in dogs that *l*-alanine, glycine, *l*-arginine and sodium chloride depress Tm_{SO_4}, conceivably because of specific inhibition of an enzyme, or because of competition for a carrier system. Evidence in favor of a carrier system was adduced by Berglund, Helander and Howe (18), who demonstrated in the dog mutual competitive inhibition between sulfate and thiosulfate reabsorption.

Gershberg, Hodler and Gasch (19) reported that a synthetic low protein diet in dogs caused an increase in the plasma sulfate concentration, possibly a result of increased tubular reabsorption. A high protein diet or DCA had no effect on tubular reabsorption, while cortisone increased the maximal rate of sulfate reabsorption. Gershberg and Gasch (20) also noted an increase in Tm_{SO_4} in three of four dogs given growth hormone. They suggested that some intermediate change in protein metabolism induced by the hormone may affect Tm_{SO_4}. No attempts were made in the present study to alter the reabsorption of sulfate in man.

SUMMARY

1. Tubular reabsorption of inorganic sulfate in man is limited by a maximal rate (Tm_{SO_4}).

2. This tubular transfer rate is not reproducible in any one individual and varies markedly between individuals.

3. The mechanism responsible for the lack of reproducibility remains unexplained.

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TRANSCORTIN: A CORTICOSTEROID-BINDING PROTEIN OF PLASMA. III. THE EFFECTS OF VARIOUS STEROIDS *†

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In previous publications (1, 2) we have described some of the characteristics of transcortin, a plasma protein (or proteins) with high affinity for cortisol and corticosterone. In addition, we have shown that plasma transcortin levels are elevated during pregnancy and following administration of estrogens (1–3). A different interpretation of the findings in these two states has been given by Daughaday and Mariz (4, 5). Transcortin levels were substantially lower in the plasma of newborn infants (umbilical vein blood) than those found in adult subjects or in older children (2). The purpose of this paper is to present results of further studies on transcortin levels in human subjects under various conditions and the effects of administered steroids on these levels.

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† The following abbreviations have been employed—cortisol: 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; dihydrocortisol (H_2F): 11β,17α,21-trihydroxy-pregnane-3,20-dione; tetrahydrocortisol (H_4F): 3α,11β,17α,21-tetrahydroxy-pregnane-20-one; 9α-fluorocortisol: 9α-fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione; cortisone: 17α,21-dihydroxy-4-pregnene-3,11,20-trione; dihydrocortisone (H_2E): 17α,21-dihydroxy-pregnane-3,11,20-trione; tetrahydrocortisone (H_4E): 3α,17α,21-trihydroxy-pregnane-11,20-dione; corticosterone: 11β,21-dihydroxy-4-pregnene-3,20-dione; aldosterone: 11β,21-dihydroxy-18-aldo-4-pregnene-3,20-dione: Compound S (11-deoxycortisol): 17α,21-dihydroxy-4-pregnene-3,20-dione; 6α-methylprednisolone: 6α-methyl-11β,17α-21-trihydroxy-1,4-pregnadiene-3,20-dione; 17α-hydroxyprogesterone (17-OHPg): 17α-hydroxy-4-pregnene-3,20-dione; Δ^4-androstenedione: 4-androstene-3,17-dione; 17α-methyl-19-nortestosterone: 17α-methyl-17β-hydroxy-4-estrene-3-one; testosterone propionate: 17β-propionoxy-4-androsten-3-one; 2α-methyl-dihydrotestosterone propionate: 2α-methyl-17β-propionoxy-androstan-3-one; estriol: 1,3,5(10)-estratriene-3, 16α, 17β-triol; 16α-estradiol dipropionate: 1,3,5(10)-estratriene-3,16α-dipropionate; Estinyl: 17α-ethinyl-3,17β-dihydroxy-1,3,5(10)-estratriene; stilbestrol: 3,4-*bis*(*p*-hydroxyphenyl)-3-hexene; Premarin: conjugated equine estrogens, containing approximately 90 per cent estrone sulfate; ACTH: adrenocorticotropin.

METHODS AND MATERIALS

The binding of C^{14}-cortisol by plasma transcortin and the transcortin capacity were determined by methods previously described (1, 2). The terms "transcortin binding of C^{14}-cortisol" and "transcortin capacity" have been defined in a previous publication (2). At this point it may be appropriate to reiterate some important aspects of the methodologies and terms used. Most of the binding experiments were performed by dialyzing 10 ml of diluted plasma (1:5 with physiological saline) against 30 ml of saline containing approximately 0.3 mg of C^{14}-cortisol. The binding was determined with and without the addition of 1 μg of cortisol to the saline in order to measure the decrease in the binding caused by the addition of the carrier cortisol. In previously published work (1) we demonstrated that under the conditions of our assay the binding of cortisol by plasma was essentially due to transcortin and not to other plasma proteins. Hence, the term "transcortin binding of C^{14}-cortisol" will refer to the percentage of C^{14}-cortisol bound by 10 ml of diluted plasma and the term "transcortin capacity" will be used to indicate the decrease in binding caused by the addition of 1 μg of cortisol in the dialysis conditions outlined above. It should be pointed out that the "binding capacity" will be inversely related to the decrease in the binding caused by the addition of 1 μg of cortisol to normal plasma, i.e., the less the decrease in the binding the higher the binding capacity, and vice versa. The measurement of the binding capacity by the method outlined above seems to offer, at the moment, the most practical and sensitive index of transcortin capacity. It should be realized, however, that this measures the difference between two variables, the level of transcortin and the level of 17-hydroxycorticosteroids (17-OHCS).

Lymph was collected from the thoracic ducts of patients with advanced cancer in ice-chilled bottles and diluted 1:5 with saline for the determination of the binding of C^{14}-cortisol. The lymph used constituted an aliquot of a 24 hour specimen, whereas the binding of plasma was determined on blood obtained between 8 and 11 a.m. The 17-OHCS levels were determined by the method of Peterson, Karrer and Guerra (6). Blood from postoperative patients was obtained in the recovery room, usually 30 to 60 minutes after completion of the operation. The control samples on the same patients were drawn

several days preoperatively. The plasma and lymph samples were frozen whenever shipped or stored. The erythrocytes were separated from the plasma by centrifugation. The plasma and the upper layer of the red blood cells were removed by aspiration. The erythrocytes were then washed three times with three volumes of physiological saline, the cells being spun down after each washing.

The C^{14}-labeled dihydro- and tetrahydrocortisone acetates were converted to the free alcohols by incubation with acetyl cholinesterase.

In the double equilibrium dialysis the technique of Daughaday, Mariz, Kozak and Biederman was followed (5, 7). Thus, under these conditions, the different plasmas (from normal subjects, patients receiving estrogens, and pregnant women), and the transcortin therein, were made to compete not only for the added C^{14}-cortisol, but also for the endogenous cortisol present in the plasmas. In addition, the binding exhibited by the same plasmas (diluted and undiluted) was determined by methods described previously (2).

RESULTS

For a better understanding of the results to be presented, it may be worthwhile to discuss briefly some of the salient features of the binding of transcortin and cortisol. In Figure 1 are shown two representative curves based on data obtained in our laboratory. At low cortisol concentrations the transcortin in normal plasma will bind the steroid almost quantitatively, resulting in the left plateau-like part of the "control" curve. When the concentration of transcortin is increased (e.g., following estrogen administration) the plateau is extended to the right, as in the "test" curve. In addition, when the concentration of cortisol is increased the resultant decrease in the binding (which we call "transcortin capacity") will be smaller in the "test" plasma than in the "control" one. With increasing concentrations of cortisol a sigmoid curve is obtained, ending in a plateau-like right shoulder of the curves. In most of the studies to be reported the determination of transcortin binding was associated with the left half of the curve, with the result that small decreases in binding, upon the addition of 1 μg of cortisol in the standard test, indicated an increased transcortin capacity. In a few studies (surgical stress, ACTH and cortisol administration) the determinations were associated with the lower right half of the curve with the result that the binding of C^{14}-cortisol in the standard test was already low and the decrease in binding upon the addition of 1 μg of cortisol was small. In this case, even though the decrease in the percentage of C^{14}-cortisol bound was small, a reduced transcortin capacity is indicated. As yet, we know little about conditions which may be accompanied by changes in the shape of the sigmoid curves, either shortening or lengthening the vertical and/or horizontal distances between the two plateaus. Until the actual concentration of transcortin can be determined or the protein isolated, this aspect of transcortin remains a perplexing problem.

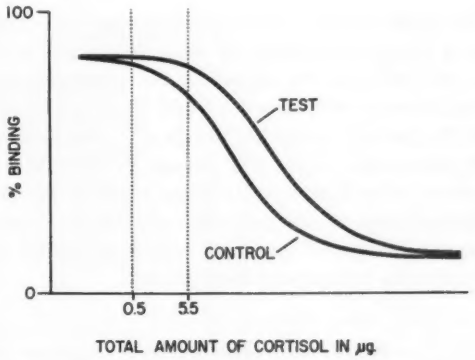

FIG. 1. CURVES FOR THE BINDING OF CORTISOL BY TRANSCORTIN. The "test" curve is applicable to plasma obtained from subjects receiving estrogen therapy and containing an increased transcortin concentration. The "control" curve applies to plasma of normal subjects. When 0.5 μg of cortisol is present, the binding by the transcortin in the "control" plasma is only slightly lower than that in the "test" plasma. At a concentration of 5.5 μg of cortisol, however, the binding by the cortisol plasma is considerably lower than that of the "test" plasma, indicating a lower transcortin capacity in the former plasma than in the latter. At very high concentrations of cortisol (right side of the curve) the differences in the transcortin binding between the two plasmas are again slight.

Transcortin levels in normal subjects and in women with cancer. During this study the transcortin binding of C^{14}-cortisol exhibited by the plasmas of 14 normal subjects was 90 ± 2.8 per cent and the transcortin capacity was 21 ± 3.3 per cent. The group of normal subjects included 7 females and 7 males (ages 22 to 45 years) with a mean plasma 17-OHCS level of 5 ± 4 μg per 100 ml. The values for transcortin binding of C^{14}-cortisol and capacity for the normal subjects in the present study are slightly higher than those reported by us previously (84 ± 12.4 per cent)

for another group of 10 normal subjects (2). The most likely explanation for these differences may be the fact that the bloods in the present study were drawn mostly between 8 and 10 a.m., whereas in the previous study the bloods were obtained in the afternoon. Since the plasma 17-OHCS are known to be higher and to vary more in the afternoon than in the morning, one might expect lower binding of C^{14}-cortisol with more spread in the binding values with such plasmas.

Three groups of postmenopausal women were studied. In a group (I) of 20 women (age range 35 to 80) with advanced breast cancer, seen by one of us in New York City, the mean values (± SD) were: transcortin binding of C^{14}-cortisol, 95 ± 3.3 per cent; transcortin capacity, 21 ± 5.6 per cent; and the plasma 17-OHCS levels, 20 ± 7 μg per 100 ml. In another group (II) of 16 women (ages 32 to 72), who had undergone surgery of the breast for cancer at least three years

TABLE I

Changes in transcortin binding of C^{14}-cortisol and capacity during estrogen and/or testosterone therapy

Subject, diagnosis	Sex, Age	Therapy	Days following beginning of therapy	C^{14}-cortisol bound	Binding capacity	Plasma 17-OHCS
				%	%	μg %
S.R.	M	Stilbestrol 5 mg 3 times	0	86	19	24
Carcinoma of prostate	59	daily from Days 1–40	0	87	25	16
			2	88	20	27
			4	92	19	26
			6	94	16	28
			8	92	11	41
			12	94	14	29
			15	94	14	32
			20	93	13	45
			25	94	11	44
			32	96	11	46
			39	98	11	40
			48	94	11	43
J.E.	F	Stilbestrol 5 mg 3 times	0	81	21	27
Hodgkin's disease	42	daily from Days 1–6	0	88	21	20
			1	90	20	32
			2	94	16	46
			3	95	15	34
			4	93	17	56
			6	98	17	44
			8	91	13	44
			10	92	15	33
			13	91	17	28
			15	91	17	24
			21	92	18	16
F.P.	F	Premarin orally 10 mg	0	86	24	11
Carcinoma of breast	54	daily for 14 days	2	90	20	11
			4	92	18	17
			7	96	12	28
			10	98	12	34
			14	98	12	36
			17	92	14	30
			21	88	20	22
			28	88	22	7
M.W.	F	Testosterone propionate	0	94	26	4
Carcinoma of breast	62	50 mg i.m. from Days	7	92	24	17
		1–14; stilbestrol 5 mg	10	99	12	13
		3 times daily from	14	99	7	33
		Days 7–14				
M.S.	F	Testosterone propionate	0	80	19	8
Carcinoma of breast	65	50 mg i.m. daily for	1	84	19	8
		5 days	3	82	21	8
			4	82	21	8
			5	92	26	
			7	87	24	13
			15	92	22	7
			18	96	25	1

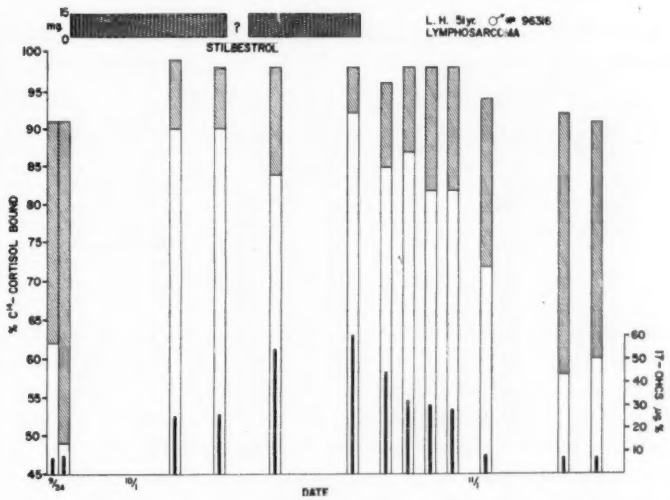

FIG. 2. CHANGES IN TRANSCORTIN BINDING OF C^{14}-CORTISOL, TRANSCORTIN CAPACITY (HATCHED AREAS), AND PLASMA 17-OHCS (BLACK BARS) DURING ESTROGEN THERAPY.

prior to the study and were apparently free of cancer, the values were: transcortin binding of C^{14}-cortisol, 94 ± 2.9 per cent; transcortin capacity, 23 ± 3.5 per cent; and the plasma 17-OHCS levels, 19 ± 5 μg per 100 ml. The values for a group (III) of 9 women (ages 39 to 74) seen in Buffalo were: transcortin binding of C^{14}-cortisol, 93 ± 3.4 per cent; transcortin capacity, 23 ± 5.1 per cent; and the plasma 17-OHCS levels, 23 ± 10 μg per 100 ml. A comparison of the various values among the groups studied revealed a statistically significant difference in the transcortin binding of C^{14}-cortisol between the normal subjects and groups I and II ($p < 0.01$) and a possible significant difference with group III ($0.02 < p < 0.05$). No significant differences were found in the transcortin capacities. The plasma 17-OHCS were higher in the patients with cancer of the breast than in normal subjects. There were no significant differences in the values found with the plasma of women with spontaneous menopause and those with surgically induced menopause.

The effects of estrogens, testosterone and other steroids on transcortin levels. It has been shown previously that the administration of adequate doses of estrogens (stilbestrol, ethinyl estradiol, Premarin) to patients causes an increase in the concentration of plasma transcortin concomitant with an increase in the plasma 17-OHCS levels (2, 3). In an attempt to find out how soon following the administration of estrogens these changes occur and for how long they persist, the transcortin levels were determined in a group of 25 patients given estrogens for varying periods of time. A few representative cases are shown in Table I and Figure 2. The estrogens administered were stilbestrol (1 to 5 mg three times daily), ethinyl estradiol (0.5 mg daily) and Premarin (2.5 to 10 mg daily). It appears that within a few days following the beginning of adequate estrogen therapy the transcortin binding of C^{14}-cortisol and transcortin capacity increased followed by a rise in 17-OHCS levels. Often there were increases in the percentage of C^{14}-cortisol bound before a change in the transcortin capacity was detectable. Thus, in 12 of the subjects the transcortin binding of C^{14}-cortisol and capacity rose by the third day. The former value rose from a mean of 90 ± 3.4 per cent to 96 ± 2.2 per cent and the latter changed from 23 ± 3.2 per cent to 16 ± 2.0 per cent. The mean value for the plasma 17-OHCS remained unchanged: 15 ± 4 μg per 100 ml. In the other subjects either no changes had occurred by the third day or the increased transcortin concentration was accompanied by rises in the 17-OHCS levels. By the fifth to seventh day the further increases in the transcortin binding of C^{14}-

TABLE II

Transcortin levels in postpartum patients

Subject	1 Hour postpartum			2 Days postpartum			5 Days postpartum		
	C^{14}-cortisol bound	Binding capacity	17-OHCS	C^{14}-cortisol bound	Binding capacity	17-OHCS	C^{14}-cortisol bound	Binding capacity	17-OHCS
	%	%	μg %	%	%	μg %	%	%	μg %
M.N.	90	16	22	95	16		98	20	7
D.M.	90	18	28	96	26	6	88	18	19
N.P.	96	8	43	94	14	36	92	15	35
M.J.				94	16	13	94	19	13
J.J.	93	16	24	86	14	43	91	15	23
V.S.	87	10	47	96	20	32	98	18	24
M.S.	93	13	47	97	4	19	95	15	30
B.T.	98	10	15	86	16	48	99	13	9
U.U.	92	14	29	94	17	22	92	17	19
M.H.	92	12	69	96	12	40	98	16	34
A.T.	92	14	80				96	24	17
B.R.	89	11	45						
B.M.							94	16	15
P.M.				98	16	15			
Mean values	92	13	41	94	16	27	95	17	20

cortisol and capacity were invariably accompanied by increases in the 17-OHCS levels in all patients studied. The increase in the transcortin concentration and that of the 17-OHCS persisted during prolonged estrogen treatment. The withdrawal of the estrogen treatment resulted in a fall in the transcortin concentration usually within a week in patients who had received stilbestrol for a short period of time, but persisted for at least 10 days to 2 weeks in patients on prolonged stilbestrol treatment.

The administration of 2.5 mg of Premarin daily for as long as 18 days failed to raise the transcortin or 17-OHCS levels. On the other hand, the daily administration of 10 mg of Premarin resulted in a definite increase in the transcortin binding of C^{14}-cortisol and transcortin capacity and elevated plasma 17-OHCS. The mean control values for five elderly females (ages 62 to 74) were: transcortin binding of C^{14}-cortisol, 90 per cent; transcortin capacity, 22 per cent; and plasma 17-OHCS, 22 μg per 100 ml. In two of the subjects 17-OHCS and transcortin levels were elevated within two days, and in all the subjects the levels were increased within a week following the daily administration of 10 mg of Premarin.

TABLE III

Transcortin levels in plasma determined on undiluted and diluted samples

Conditions	Undiluted		Diluted (1:5)			
	Sample 1	Sample 2	Sample 1		Sample 2	
	C^{14}-cortisol bound		C^{14}-cortisol bound	Binding capacity	C^{14}-cortisol bound	Binding capacity
			%	%	%	%
Normal plasma	98	97	92	20	94	28
Pregnancy plasma	98	98	95	11	98	14
Normal (N) vs pregnancy (P)	N 99	97	90	20	96	22
	P 99	99	95	11	98	15
Normal plasma	98	97	90	27	94	28
Estrogen plasma	98	99	98	11	99	8
Normal vs estrogen (E)	N 99	97	92	20	98	24
	E 99	99	96	7	99	10
Pregnancy vs estrogen		P 98			99	15
		E 99			99	9

TABLE IV

The effects of dilution of plasma on transcortin binding of C^{14}-cortisol and capacity

Subject	Conditions	Diluted (1:5)		Diluted (1:10)		17-OHCS Plasma
		C^{14}-cortisol bound	Binding capacity	C^{14}-cortisol bound	Binding capacity	
		%	%	%	%	*μg/100 ml*
S.R.	Normal	86	19	72	30	24
R.S.	Normal	87	25	74	29	16
L.P.	Leukemia	78	27	62	36	30
H.S.	Estrogen-treated	99	12	96	20	55
R.F.	Pregnancy	98	10	97	14	34

The highest transcortin binding of C^{14}-cortisol and transcortin capacity were 98 and 12 per cent, respectively. The highest 17-OHCS level was 46 μg per 100 ml. Elevation of plasma transcortin and 17-OHCS levels were seen in all patients receiving stilbestrol or ethinyl estradiol in the dosage employed. Three patients with cancer of the breast received 16α-estradiol dipropionate (5 mg three times weekly i.m.) and no changes were seen either in the transcortin or 17-OHCS levels. The administration of 3,4-*bis*(3-allyl-4-hydroxyphenyl)-hexane, a weak estrogen, in doses of 15 to 30 mg orally daily to three postmenopausal women (ages 36 to 49), did not change the transcortin binding or the 17-OHCS levels.

The administration of 17α-methyl-19-nortestosterone (30 mg daily orally) also failed to cause changes when administered for as long as 36 days. The administration of testosterone propionate (100 mg i.m. three times weekly) to three subjects failed to change the transcortin and 17-OHCS levels significantly, even when administered for as long as 76 days. The administration of large doses (100 mg i.m. three times weekly) of testosterone propionate to 15 additional subjects, including 10 postmenopausal women with cancer of the breast, and 2α-methyl-dihydrotestosterone propionate (100 mg three times weekly) to three subjects failed to change the transcortin levels significantly. In addition, Patient M. W. (Table I) while on tesosterone therapy received estrogens and, as can be seen, the transcortin levels and the 17-OHCS rose. In Patient M. S. the transcortin binding of C^{14}-cortisol rose somewhat during testosterone therapy, but the transcortin capacity and 17-OHCS in the plasma remained normal or decreased.

Transcortin levels postpartum. Transcortin levels have been shown to be elevated during pregnancy, especially during the last trimester (1).

TABLE V

The effects of surgery on transcortin and 17-OHCS levels

Subject	Control			Postoperative		
	C^{14}-cortisol bound	Binding capacity	17-OHCS	C^{14}-cortisol bound	Binding capacity	17-OHCS
	%	%	μg %	%	%	μg %
U.K.*	94	23	21	78	16	25
				64	1	24
K.L.	88	26	12	74	20	30
M.Ho.	92	23	10	71	16	26
E.B.	88	20	16	72	9	34
M.Ha.	90	25	21	75	11	34
J.W.	91	23	5	77	16	24
D.O.	99	24	15	72	18	37
E.D.	87	25	9	70	19	34
R.C.	90	32	7	58	4	64
N.N.	85	23	15	68	12	52
N.P.	84	24	10	69	16	43
J.A.	86	22	33	81	24	29

* The levels in Subject U.K. were determined following two separate surgical procedures performed on different occasions.

TABLE VI

The effects of various in vitro cortisol concentrations on the binding of C^{14}-cortisol (expressed as per cent bound) by plasma from normal and postoperative subjects and by HSA

Cortisol (μg)	Normals		Operated subjects				1% HSA
	1	2	3	4	5	6	
0.3	80 [19]*	89 [10]	58 [64]	72 [37]	68 [52]	81 [29]	22
1.3	55	66	54	54	56	57	22
2.3	49	48	52	42	54	48	21
4.3	44	38	48	26	36	36	20
7.3	40	34	44	22	34	22	19
11.3	35	31	42	19	29	19	18
15.3	32	30	36	20	29	12	18
21.3	26	26	36	20	29	10	18
			Preoperative levels				
0.3			90 [7]	88 [15]	85 [15]	86 [33]	
1.3			58	64	62	64	

* Values in brackets are the 17-OHCS levels in μg%.

The transcortin binding of C^{14}-cortisol and capacity were determined in a group of 14 women 1 hour, 2 days and 5 days postpartum, as well as 6 weeks thereafter (Table II). The transcortin capacity in the immediate postpartum samples continued to be elevated, accompanied by very high 17-OHCS levels in some of the patients. By the fifth day postpartum the values for the transcortin binding of C^{14}-cortisol and capacity had changed little, but the 17-OHCS concentration had decreased 50 per cent when compared to the values obtained 1 hour postpartum. By the sixth week all values had reached normal concentrations. The mean values (± SD) determined in the plasmas of 18 patients (including the 14 patients studied above) at 6 weeks postpartum were: transcortin binding of C^{14}-cortisol, 91 ± 3.2 per cent; transcortin capacity, 24 ± 3.9 per cent; and plasma 17-OHCS, 9 ± 6.2 μg per 100 ml.

Comparison of methods. A comparison of the binding of C^{14}-cortisol by plasmas from normal subjects, pregnant women, and subjects treated with estrogens was made by two different techniques. Daughaday and associates (7, 8) in their studies failed to find a difference between the first two, using a method somewhat different from ours (undiluted plasma). Accordingly, we determined the binding of the same plasmas by both techniques. Samples 1 and 2 in Table III represent two separate sets of experiments performed with plasmas from different subjects in each experiment. As can be seen (Table III), no difference was observed in the binding of plasmas using Daughaday's method of double dialysis, but defi-

TABLE VII

The effects of ACTH and steroids on the transcortin and 17-OHCS levels

Subject	Substance administered	Control			Time following administration	Post-administration		
		C^{14}-cortisol bound	Binding capacity	17-OHCS		C^{14}-cortisol bound	Binding capacity	17-OHCS
		%	%	μg %		%	%	μg %
C.L.	ACTH	88	16	27	6 hrs	69	5	49
D.S.	ACTH	65	12	69	6 hrs	47	1	161
D.Sc.	ACTH	88	21	14	6 hrs	74	10	46
H.E.	Intravenous cortisol	82	22	17	2 hrs	65	11	66
					4 hrs	55	0	69
					6 hrs	53	0	118
A.S.	4 mg 9α-Fluorocortisol daily	86	16	6	3 days	92	22	1
					4 days	90	21	1
S.G.	1 mg 9α-Fluorocortisol i.v. over 8 hrs	94	24	11	8 hrs	92	19	10

nite differences were shown with the use of our method. Even though differences were observed in the transcortin binding of C^{14}-cortisol, the most striking differences were seen in the transcortin capacity. The determination of C^{14}-cortisol binding and transcortin capacity on plasmas diluted 1:10 (instead of the usual 1:5) revealed a definite difference between the plasmas of a pregnant woman and a subject receiving estrogens on one hand, and between those of two normal subjects and a leukemic patient on the other (Table IV).

The effect of surgical stress on transcortin levels. It can be observed that the binding of C^{14}-cortisol and transcortin capacity decreased considerably following surgery, concomitant with a rise in plasma 17-OHCS (Table V). The exception was Patient J. A. who did not show a rise in the plasma steroids following a 1 hour surgical procedure; no change of significance was discerned in transcortin levels. To test further the reduced C^{14}-cortisol binding and transcortin capacity accompanying the rise in plasma 17-OHCS in postoperative patients, the effects of increased *in vitro* concentrations of cortisol on the binding of C^{14}-cortisol (1) were studied on the plasmas of normal subjects and postoperative cancer patients and in a solution of 1 per cent human serum albumin (HSA).

The latter determinations were performed in order to ascertain the binding exhibited by the HSA, which is thought to be the major binding protein of cortisol when transcortin becomes saturated with the steroid. The results in Table VI show that with increasing concentrations of cortisol the binding of C^{14}-cortisol decreased, following in general the pattern shown in Figure 1. The binding of C^{14}-cortisol exhibited by HSA is small but of high capacity, as evidenced by the low decreases in binding with increasing concentrations of cortisol. As can be seen (Table VI), binding capacity was decreased in postoperative patients (see first paragraph under Results).

TABLE VIII

The binding of certain C^{14}-steroids by human plasma (diluted 1/5 with saline) and albumin (values refer to per cent of C^{14}-steroid bound)

Steroid	Plasma vs 1% HSA	Plasma vs saline	1% HSA vs saline
H_4E	25	52	48
H_2E	48	53	29
17α-Hydroxyprogesterone	90	98	86
Δ^4-Androstenedione	4	84	86
6α-Methylprednisolone	61		
Aldosterone	62 [4]*	74 [40]	18
	52 [1]	60 [32]	

* Figures in brackets refer to the binding of C^{14}-aldosterone caused by the addition of 1μg of cortisol.

TABLE IX

*The effects of the addition of various corticosteroids on the binding of C^{14}-cortisol**

Steroid added	H_2F	H_2E	Aldosterone	Compound S	17-OHPg	Corticosterone
μg						
1	97	98	90	40	46	72
3	96	96				
5	95	94	79			
10	92	90	70			
20	89	87				

* Values refer to the percentage of C^{14}-cortisol bound and represent the mean of at least four determinations. The binding of C^{14}-cortisol exhibited by the plasma was 97 per cent, with a transcortin capacity of 27.

Effects of ACTH and corticosteroids. Administration of ACTH intravenously (40 units over a period of 6 hours) to patients with cancer resulted in elevated 17-OHCS, causing a decreased C^{14}-cortisol binding and transcortin capacity (Table VII). The same was true when a patient with cancer was given cortisol intravenously. The small changes observed in the binding upon the addition of 1 μg of cortisol, seen in some patients with cancer given ACTH or following surgery, in face of a fairly high percentage of C^{14}-cortisol bound, are of interest. The administration of 9α-fluorocortisol resulted in insignificant changes in transcortin binding of C^{14}-cortisol and transcortin capacity. One might not expect a change in the binding of C^{14}-cortisol or in the transcortin capacity when the 17-OHCS levels decrease slightly or do not change, as occurred following the administration of 9α-fluorocortisol.

In Table VIII is shown the binding by plasma or 1 per cent HSA of various C^{14}-steroids. In addition, plasma was dialyzed against HSA in order to cancel the effects of the plasma albumin on the binding (1). This procedure showed that Δ^4-androstenedione is predominantly bound to the serum albumin and that transcortin shows little affinity for the dihydro (H_2E) and tetrahydro (H_4E) derivatives of cortisone, aldosterone and 6α-methyl prednisolone. In the case of C^{14}-aldos-

TABLE X

Transcortin levels in lymph and plasma of subjects with cancer

Subject	Lymph			Plasma		
	C^{14}-cortisol bound	Binding capacity	17-OHCS	C^{14}-cortisol bound	Binding capacity	17-OHCS
	%	%	μg %	%	%	μg %
B.I.	64	25	15	81	15	31
N.S.	76	16	17	80	16	22
R.B.	80	27	14	78	13	
L.Y.	58	18	13	75	17	
L.N.	62	18	10	76	20	9
L.M.	64	20	15			
W.M.	70	24	10	76	16	18
L.A.	91	15	23	96	15	9
A. W.	74	20	22	78	24	6
E.S.	63	13	28			
A.F.	64	31	9			
C.L.	86	38	10			
B.H.	82	36	14			
D.R.	62	44	11			
	66	30	19			
Mean values	71	25	15	80	17	16

terone, added cortisol easily displaced the former from its binding sites. As can be seen from Table VIII, of all the steroids studied, only 17α-hydroxyprogesterone (17-OHPg) was bound to the same extent by plasma as is cortisol but was bound more extensively by human serum albumin than was cortisol. Competition experiments between various steroids, including some of the major metabolites of cortisol, revealed that only corticosterone, compound S (11-deoxycortisol) and 17α-hydroxyprogesterone are good competitors for the binding sites (Table IX). One μg of estriol or of cortisone failed to change the binding of C^{14}-cortisol using the standard test. When tetrahydrocortisol or tetrahydrocortisone was added in concentrations of 1 to 20 μg, no change in the binding of C^{14}-cortisol occurred. The dihydro derivatives of cortisol or cortisone and aldosterone had a minor effect when present in very large concentrations.

The binding of C^{14}-cortisol by lymph. The determination of C^{14}-binding and transcortin capacity was performed on lymph obtained from the thoracic ducts of patients with advanced cancer. From Table X it can be seen that the lymph, on the average, contained transcortin and 17-OHCS in concentrations somewhat lower than those seen in the plasmas of the same subjects.

The mean concentration of total protein in ten lymph fluids was 3.35 g per 100 ml. Examination of these fluids by paper electrophoresis revealed the following mean values: 33 per cent of albumin, 12 per cent of α_1-globulin, 11 per cent of α_2-globulin, 16 per cent of β-globulin, and 28 per cent of γ-globulin. The mean total protein concentration in the sera of these patients was 5.92 g per 100 ml. Paper electrophoretic analysis of the sera revealed 39 per cent albumin, 9 per cent α_1-globulin, 13 per cent α_2-globulin, 20 per cent β-globulin, and 19 per cent γ-globulin.

The binding of C^{14}-cortisol by erythrocytes. A study was made of the binding of C^{14}-cortisol by erythrocytes in subjects given estrogens, in order to determine whether the increased binding exhibited by the plasma is also shown by the red blood cells. The binding was determined by dialyzing a 10 per cent suspension of red blood cells against saline. Even though there was an increase in the binding of C^{14}-cortisol (control binding 0 to 10 per cent) to as high as 46 per cent, the addition of 1 μg of cortisol abolished all the binding of C^{14}-cortisol (0 to 4 per cent).

Transcortin changes in a subject with Addison's disease. A subject with known Addison's disease and with no demonstrable 17-OHCS levels in his blood or urine was taken off cortisone therapy for a period of 4 days. The patient was then given stilbestrol (1 mg three times daily for 4 days) and the transcortin binding of C^{14}-cortisol and capacity were determined. The binding of C^{14}-cortisol by the control (before estrogen administration) plasma was 98 per cent, with a transcortin ca-

pacity of 22 per cent, whereas following the administration of the estrogen the binding was 98 per cent, with a capacity of 6 per cent, indicating a definite effect of estrogen on the transcortin concentration in this patient. The 17-OHCS levels remained at zero during the stilbestrol administration.

DISCUSSION

The data of the present study extend our observations on the effect of estrogens on the plasma transcortin levels in human subjects. The results indicate that rises in transcortin binding and capacity precede the elevation of 17-OHCS in the plasma, suggesting that the increases in the corticosteroid levels observed in conditions with high transcortin levels (pregnancy, estrogen administration) may be due to the inability of the body to metabolize transcortin-bound cortisol with resultant increase in plasma corticosteroid levels. The rises in the transcortin levels induced by estrogens occurred as early as 3 days after commencement of therapy and, although observed with all known active estrogens given, they depended on the dose of the steroid administered. Thus, 2.5 mg of Premarin daily failed to induce a rise, whereas 10 mg did. On the other hand, as little as 3 mg daily of stilbestrol or 0.5 mg daily of ethinyl estradiol produced definite elevations of transcortin levels. It should be pointed out that on the basis of bioassay the following equivalents have been shown: 1.25 mg of Premarin has the activity of 600 to 800 rat units, 1 mg of stilbestrol, 1,500 rat units, and 0.1 mg of ethinyl estradiol, 1,200 rat units (9). From the data shown it would appear that a minimum of 5,000 rat units per day must be administered in order to induce a demonstrable increase in transcortin concentration. The increased transcortin levels returned to normal concentrations within 1 to 2 weeks following cessation of effective estrogen therapy, with the 17-OHCS concentrations declining to normal levels soon after. Herrmann, Schindl and Bondy (10) have recently reported the failure of 5 mg of Premarin to significantly alter levels of plasma cortisol or their response to ACTH infusion, while it significantly lowered urinary excretion of adrenal steroids. These authors implied that the decreased catabolism of cortisol may be due to increased amounts of protein binding of cortisol in plasma. These observations are borne out by some of the data presented in this paper and by previous observations (1–3). In addition, recently published data (3, 11, 12) substantiate the hypothesis advanced by us (1, 2) that the changes in cortisol metabolism seen during estrogen administration are due to changes in the plasma levels of transcortin.

The values, higher than those seen in normal subjects, observed for the transcortin binding of C^{14}-cortisol in postmenopausal women with various stages of cancer of the breast may be due to the higher concentrations of transcortin, especially when considered in conjunction with the higher plasma 17-OHCS observed in these patients. At present it is difficult to state whether these increases are due to age, sex, or the disease of the patients. Further studies are necessary for clarification of this point. It is interesting to note that elevated levels of serum protein-bound iodine and possibly of serum thyroxine-binding protein have been observed in patients with progressive metastatic carcinoma of the breast (13).

In pregnant women the transcortin levels and the 17-OHCS continued to be elevated 5 days postpartum but returned to normal 6 weeks postpartum.

Of the many steroids studied, only the known active estrogens were capable of inducing concomitant increases in transcortin and 17-OHCS levels. The changes in transcortin capacity induced by estrogens in an Addisonian subject without any demonstrable plasma or urinary 17-OHCS would seem to indicate that adrenocortical function is not necessary for the estrogens to cause increased concentrations of transcortin. The binding of C^{14}-cortisol by erythrocytes *in vivo* was similar to that previously described (14) and was not greatly changed by the administration of estrogens, even though the slight increase in the binding observed may be due to some trapped plasma or binding of small amounts of transcortin to the red blood cells.

Testosterone propionate, 17α-methyl-19-nortestosterone, and 2α-methyl-dihydrotestosterone propionate were incapable of producing any significant changes in the concentrations of transcortin or 17-OHCS. In addition, testosterone was not able to prevent the changes in transcortin concentration induced by estrogen therapy while the patient was

receiving large doses of testosterone. In a few instances slight increases were seen during therapy with very large doses of testosterone, changes which may possibly be due to conversion of the androgen to estrogens, as has been reported by others (15, 16). The failure of steroids other than estrogens to cause a rise in the transcortin levels points to a specificity of action of these steroids. This is of note, especially in view of the finding that during the administration of large doses of testosterone, stilbestrol was able to cause an increase in the transcortin capacity.

Rises in the levels of 17-OHCS induced by ACTH, stress, and infusion of cortisol caused great decreases in transcortin binding of C^{14}-cortisol and in transcortin capacity due to saturation of the protein with cortisol, which probably occurred at levels of 20 to 40 μg per 100 ml. With high plasma 17-OHCS levels, the conditions prevailing for the binding of cortisol and transcortin are best depicted in the right half of the "control" curve in Figure 1. As transcortin becomes saturated with cortisol, the percentage of the steroid bound is greatly reduced and additional cortisol causes only small decreases in the amount of steroid bound. The small changes in the C^{14}-cortisol bound upon the addition of 1 μg of cortisol seen in some patients with cancer given ACTH or following surgery, in face of a fairly high percentage of C^{14}-cortisol bound before the addition of the extra cortisol, may be due to several factors. It is possible that under the stress of surgery or due to ACTH administration the concentration of transcortin or some other corticosteroid-binding protein increases in the plasma. Furthermore, surgery may result in the appearance in the blood of tissue proteins with affinity to cortisol, akin to the thyroxine-binding protein present in muscle tissue. In addition, surgery or ACTH may induce in cancer patients a shift in the ratio of the various plasma proteins, resulting in an abnormal pattern of steroid-protein interaction.

It is interesting that the unconjugated metabolites of cortisol, dihydro-(H_2E) and tetrahydrocortisone (H_4E), and dihydro-(H_2F) and tetrahydrocortisol (H_4F) and aldosterone, did not impinge upon the binding capacity of cortisol unless they were present in excess of physiological levels ordinarily found in plasma. Similarly, when tetrahydrocortisol or tetrahydrocortisone was administered intravenously, the transcortin binding of C^{14}-cortisol and transcortin capacity did not change in spite of the greatly elevated plasma 17-OHCS levels (17). Only corticosterone, 11-deoxycortisol (compound S) and 17α-hydroxyprogesterone seem capable of effectively competing with cortisol for the binding sites on transcortin. From the results in Table IX it appears that steroids of the pregnane series with a Δ^4-3-keto group and a 17α-hydroxy-20-keto group have great affinity for transcortin and that an 11β-hydroxyl, and especially 11-keto, group may actually interfere with the binding. These results confirm, in essence, the findings of Daughaday and Mariz (5) regarding the inhibition of the binding of C^{14}-cortisol by various steroids. Since corticosterone and 11-deoxycortisol are present in much lower concentrations than cortisol, and since 17α-hydroxyprogesterone has not been demonstrated in human blood, it would appear that under normal conditions transcortin primarily binds cortisol.

The appearance of transcortin in lymph might be due to transcortin which may have left the circulation and is returning via the lymph or it may be due to synthesis of transcortin in a system drained by the lymph. It is interesting to note that the concentration of α_1-globulin in the lymph was of about the same magnitude as that in the serum of the cancer patients. Since transcortin is found in the α_1-globulin fraction, it is not surprising to find that the binding exhibited by the lymph fluids was not much lower than that found with plasma. Even though some of the other globulins were present in higher concentrations in the lymph than in the serum, these proteins have been shown to have little affinity for cortisol (14). It should be pointed out, however, that the lymph in our studies was obtained from patients with advanced cancer and these findings may not be applicable to normal subjects. The possible presence of transcortin in some body fluids (synovial, pleural) and its absence in spinal fluid is indicated in the work of Daughaday and co-workers (7).

Daughaday and associates, in earlier work (7, 8), failed to find a difference in the binding of labeled cortisol between normal plasma and that obtained from pregnant women or subjects receiving large doses of estrogens. They utilized undiluted plasma in equilibrium dialysis and in a competition or double dialysis, wherein plasma from

pregnant women or subjects receiving estrogens was dialyzed in the same system with normal plasma, but definite differences were demonstrated, using their system of dialysis as well as ours, when diluted plasma was used. Again, we attributed Daughaday's failure to show a difference between normal and pregnant plasma, and plasma of estrogen-treated subjects, to the high equilibrium constant of transcortin associated with the use of a high protein concentration.

Daughaday and Mariz's conclusion, based on more recent work (5), that there are two corticosteroid-binding globulins in the plasma of pregnant women or of subjects treated with estrogens, is open to another interpretation. They "compared the binding of cortisol by four plasmas of individuals with increased estrogenic activity directly to control plasma using (their) standard conditions (double equilibrium dialysis) of cortisol loading (0.5 μg per 10 ml of plasma) and also using 5.5 μg of cortisol per 10 ml of plasma." The *ratio* of the binding of cortisol by the plasma of test subjects to that of the control subjects increased with the increased cortisol load. Daughaday states that "in such a situation the relative binding of cortisol would be greatest at the lowest level of cortisol loading." This is not the case, however. Let us consider the two sigmoid binding curves shown in Figure 1. At 0.5 μg the binding of the two plasmas is nearly identical, but at 5.5 μg the *ratio* of binding is considerably larger than unity. Thus, Daughaday is observing the same phenomenon as we, although expressing it differently. Indeed, we are in complete accord on the facts, which in our opinion, point to the inability of any of the published methods to distinguish two corticosteroid-binding globulins in human plasma.

SUMMARY

The administration of adequate amounts of estrogens produces increased concentrations of transcortin within 3 to 7 days, followed by a rise in the levels of the 17-hydroxycorticosteroids (17-OHCS). These levels declined to normal within 7 to 10 days after cessation of estrogen treatment. The increased concentration of transcortin can be induced by estrogens in the absence of the adrenal gland.

Testosterone propionate, 17α-methyl-19-nortestosterone and 2α-methyl-dihydrotestosterone propionate failed to change the transcortin or 17-OHCS concentrations.

The increased transcortin concentrations in pregnant women had changed little within 5 days postpartum and were normal 6 weeks after delivery.

ACTH, surgical stress, and intravenous cortisol decreased the transcortin binding of C^{14}-cortisol and transcortin capacity.

Lymph fluids contain transcortin and 17-OHCS in slightly lower concentrations than those seen in plasma.

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ADRENAL MEDULLARY INSUFFICIENCY IN SEVERE THERMAL BURN *

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Although adrenal medullary insufficiency is occasionally seen in Addison's disease and under certain experimental conditions (1–3), it has not been described in man as a result of thermal burns or of other clinical states.

The normal human adrenal contains 412 to 633 μg of adrenaline and 37 to 123 μg of noradrenaline. These results compare favorably with those of von Euler, Franksson and Hellström (4). Adrenaline is the principal hormone of the adrenal medulla (4–6) and is released in increased amounts under various stressful situations, i.e., thermal burns (7), muscular exercise (8), centrifugation (9), and trauma (10). Noradrenaline, although also found in the human adrenal medulla (4, 6), is primarily derived from the sympathetic nerves (11–16). Noradrenaline is the neurohormone of the sympathetic nerves and is released as such at the adrenergic nerve endings (12, 14, 16). Like adrenaline, noradrenaline is also liberated in increased amounts under various stressful situations (7–10).

Both of these hormones, as well as their metabolic products (17–21), are excreted in the urine in amounts which parallel the sympathetic nerve and adrenal medullary activity (6). Normal young adult males excrete approximately 10 to 25 μg of adrenaline and 25 to 45 μg of noradrenaline per 24 hours (7, 22). It is further known that the adrenal gland normally synthesizes adrenaline with such rapidity (23–25) that only under unusual circumstances can the adrenal medulla be depleted of its adrenaline supply (1, 3). It has been shown that most patients who survive severe thermal burns excrete daily large amounts of adrenaline and noradrenaline, and that this elevated rate of excretion continues for weeks without any recognizable failure in the adrenal gland's ability to produce adrenaline (7). However, of the burned patients who died, approximately two-thirds showed a markedly depressed adrenaline output at the time of death. The experiments herein described attempt to correlate this low adrenaline output with a low adrenaline content of the adrenal gland and further, to evaluate this finding in terms of an acute adrenal medullary insufficiency.

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METHOD

Urine

A. General. Daily 24-hour urines were collected and bioassayed for adrenaline and noradrenaline.

B. Preparation of urine extract. The procedure was adopted from von Euler and Hellner (22). The urine was hydrolyzed and the adrenaline and noradrenaline selectively adsorbed on aluminum hydroxide and filtered. The precipitate was washed and redissolved with 2 N H_2SO_4. The remaining salts were precipitated out by mixing the extract with alcohol and acetone. The filtrate was concentrated *in vacuo*.

C. Biological assay of urine. This procedure has been previously described (4, 5, 7, 26). In brief, the cat's blood pressure, which is sensitive to noradrenaline, is used in conjunction with the fowl's rectal cecum, which is sensitive to adrenaline.

The cat's blood pressure was recorded from the carotid artery and injections of adrenaline, noradrenaline and urinary extract were made into the femoral vein.

The fowl's rectal cecum was suspended in a water bath containing Tyrode's solution at 39° C. Through the Tyrode solution, 6.5 per cent of CO_2 in oxygen was bubbled. Injections of adrenaline, noradrenaline and urinary extract were made into the bath and the degree of cecal relaxation was recorded on a kymograph.

D. Computation of results. Having determined the activity ratio for adrenaline and noradrenaline on the cat's blood pressure and on the hen's rectal cecum, and the activity of the unknown urinary extract in terms of *l*-noradrenaline, it is possible to calculate the relative amounts of adrenaline and noradrenaline in the urinary extract (4, 5, 7, 26).

Adrenal gland

A. General. The adrenal gland was removed 1 to 7 hours after death, weighed, and placed in 5 per cent trichloroacetic acid.

B. Preparation of adrenal extract. The procedure used for preparing the extract is rather similar to that described under "urine extract." However, as a prelimi-

TABLE I

Urinary adrenaline and noradrenaline of burned patients who died with low (Group A) and high (Group B) adrenaline output

	Calculated 24-hr output of adrenaline and noradrenaline							
	Group A		Group B					
	J.J., ♂, 44 yrs, 50% burn, 25% 3° burn		J.H., ♀, 12 yrs, 88% burn, 65% 3° burn		R.C., ♂, 65 yrs, 38% burn, 33% 3° burn		M.W., ♀, 25 yrs, 95% burn, 90% 3° burn	
No. of days after burn	Adr.	Nor.	Adr.	Nor.	Adr.	Nor.	Adr.	Nor.
Control*	15.7	32.3	15.7	32.3	15.7	32.3	15.7	32.3
1	118.3	111.5			50.9	187.2	253.6	46.3
2	82.8	176.2	Delay in				234.5	24.2
3	42.8	183.0	entering		48.3	165.6	159.2	91.3
4	19.6	98.0	hospital		111.5	78.1	119.7	104.1
5	16.6	83.0			140.3	161.5		
6	44.1	160.0						
7	33.4	104.4			30.2	226.5		
8								
9								
10			91.0	119.9	25.6	161.4		
11			112.0	113.8				
12			184.6	139.1	70.1	287.2		
13			183.1	141.3	87.8	280.8		
14			93.6	138.8				
15								
16			152.5	199.3				
17								
18			224.3	253.7				
Patient adrenals (μg/g)	594.3	89.5	Destroyed		23.5	0.0	27.5	9.0
Normal adrenals (μg/g)								
Adrenaline: 412–633								
Noradrenaline: 37–123								

* Control represents average normal 24-hour urinary output of adult males.

nary step, the adrenal gland is homogenized in trichloroacetic acid and the adrenaline and noradrenaline are then selectively adsorbed on aluminum hydroxide and subsequently eluted.

C. Colorimetric assay. Because of the large quantities of adrenaline normally found in the human adrenal gland, the colorimetric method of von Euler and Hamberg (*27*) was used. This method is based upon the different oxidation velocity of adrenaline versus noradrenaline with iodine at pH 4 and pH 6; adrenaline is completely oxidized in 1.5 minutes at pH 4, while only 10 per cent of noradrenaline is oxidized at this pH. At pH 6, both amines show complete oxidation.

Rate of adrenaline deterioration following death

In order to determine the amount of deterioration of adrenaline that might occur in the adrenal gland following death, 12 rabbits were killed and maintained at room temperature. Then, at hourly intervals for 12 hours following death, the adrenals of one rabbit were removed and assayed for adrenaline and noradrenaline, thereby affording an hourly deterioration picture of adrenaline in the adrenal gland. The results of these experiments showed that no appreciable destruction of either adrenaline or noradrenaline took place within a 10 hour period following death but, thereafter, there was a significant decline in both of these hormones. These findings would seem to indicate that as long as the adrenal glands were removed from the patient within 10 hours after death, very little destruction of adrenaline would be expected.

RESULTS

A total of 14 severely burned patients was followed, all of whom died. In each patient, the daily 24-hour urinary output of adrenaline and noradrenaline was determined and at death, correlated to the adrenaline content of the adrenal gland.

One of the 14 patients (J.J.) showed at first a markedly elevated adrenaline output, but this gradually declined until, at the time of death, the output was approximately normal but low relative to the degree of stress. The adrenaline content of the adrenal gland of this patient was normal (Table I, Group A).

Two other patients (R.C. and M.W.) showed a high output of adrenaline throughout the burn period. The adrenaline content of their adrenal glands was greatly reduced (Table I, Group B).

The remaining 10 patients, i.e., 71.5 per cent of

all the burned patients, showed at the time of death a low output of adrenaline which was subnormal for the stress imposed by the burn. The adrenaline content of the adrenal gland was greatly reduced and in some instances (E.B. and H.T.) approached zero. However, it should be noted that during the first 3 or 4 days after the burn, most of the patients in this group excreted large quantities of adrenaline, but this gradually decreased so that at the time of death the output was subnormal (Table II).

The normal urinary levels of adrenaline and noradrenaline were determined from the 24-hour specimens of normal adult males varying in age from 20 to 40 years. Calculated on the basis of 24-hour output, the average quantity of adrenaline excreted was 15.7 μg, and of noradrenaline 32.3 μg. These results were used as controls and are similar to the normal output as determined by von Euler and Hellner (22).

In normal subjects, the urinary output of adrenaline and noradrenaline is independent of the urine volume. This would seem also to hold true in these burn patients, although some of them showed signs of acute renal failure with oliguria, elevated creatinine excretion, and an elevated blood urea nitrogen (BUN). However, as long as the patient's 24-hour urinary output exceeded 150 ml, the renal failure apparently had little or no effect upon the urinary output of adrenaline and noradrenaline. This can be seen from Patient W.V. who on the first day after burn showed a urinary output of 220 ml, and on the terminal day an output of 173 ml; however, the urinary output of adrenaline for these days was 658.2 and 19.1 μg, respectively (Table II). Also, there were several patients (J.J., H.T. and J.L.) who terminally showed a low adrenaline output but a urine output in excess of 680 ml per 24 hours and a BUN of 16 to 92 mg per 100 ml.

TABLE II

Urinary adrenaline and noradrenaline of burned patients who died with low adrenaline output and low adrenaline content of the adrenal gland

	Calculated 24-hr output of adrenaline and noradrenaline									
	J.W., ♂, 32 yrs, 81% burn, 50% 3° burn		R.T., ♂, 27 yrs, 60% burn, 50% 3° burn		A.W., ♂, 19 yrs, 85% burn, 59% 3° burn		E.T.L., ♀, 49 yrs, 47% burn, 35% 3° burn		J.M., ♂, 43 yrs, 75% burn, 75% 3° burn	
No. of days after burn	Adr.	Nor.	Adr.	Nor.	Adr.	Nor.	Adr.	Nor.	Adr.	Nor.
Control*	15.7	32.3	15.7	32.3	15.7	32.3	15.7	32.2	15.7	32.3
1	125.3	303.4	42.0	274.9	257.1	113.9	34.0	20.6	394.0	121.6
2	62.1	230.4	58.1	313.1	54.8	229.0	10.1	22.6	142.1	226.6
3	54.6	374.9	62.9	322.6	30.4	71.3	3.1	22.9	32.9	237.2
4	18.8	152.5	63.6	302.4			7.5	5.6	210.1	280.1
5	3.7	30.0	78.3	213.9					74.0	323.5
6			40.8	249.9					67.3	117.7
7			80.8	158.1					33.0	152.7
8			49.1	172.8					43.0	184.2
9			19.9	62.6					79.6	77.9
10			1.9	7.5					13.5	95.2
Patient adrenals (μg/g)	150.8	49.9	141.9	38.6	118.7	44.3	294.0	112.8	191.0	36.7
	W.V., ♂, 29 yrs, 90% burn, 50% 3° burn		F.T., ♂, 51 yrs, 85% burn, 85% 3° burn		J.L., ♂, 39 yrs, 94% burn, 70% 3° burn		E.B., ♀, 47 yrs, 55% burn, 50% 3° burn		H.T., ♂, 35 yrs, 83% burn, 82% 3° burn	
	Adr.	Nor.	Adr.	Nor.	Adr.	Nor.	Adr.	Nor.	Adr.	Nor.
Control*	15.7	32.3	15.7	32.3	15.7	32.3	15.7	32.3	15.7	32.3
1	658.2		13.0	22.0	41.5	54.1	48.9	71.4	52.5	14.0
2	74.8	51.9			24.6	71.4	11.9	74.2	152.9	274.4
2	47.9	130.8			19.4	108.2	8.4	66.3	46.4	126.7
4	19.1	73.2			20.2	73.9	3.7	47.6		
5					12.9	22.6				
Patient adrenals (μg/g)	63.9	46.0	86.8	179.0	50.8	18.0	8.5	12.9	7.8	13.5
Normal adrenals (μg/g)										
Adrenaline: 412–633										
Noradrenaline: 37–123										

* Represents average normal 24-hour urinary output of adult males.

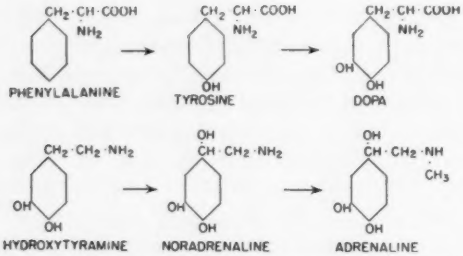

FIG. 1. BIOSYNTHETIC PATHWAY TO THE FORMATION OF ADRENALINE AND NORADRENALINE.

DISCUSSION

From the results described in Tables I and II, it is apparent that these burned patients at the time of death fall into three distinct groups: Group A, low to normal adrenaline output with normal adrenaline content of the adrenal gland; Group B, high adrenaline output with a low adrenaline content of the adrenal gland; and Group C, low adrenaline output with a low adrenaline content of the adrenal gland. In terms of supply and demand for adrenaline, these three groups would appear to be gradations of the same conditions, that is, the supply or synthesis of adrenaline did or did not keep up with the demand for and release of this hormone. The demand is represented by the physiological "stress" of the burn.

The low to normal adrenaline output with normal adrenals, as seen in Table I, Group A, represents those patients in whom the severity of burn was considerably less than that observed in Table II, Group C. The demand for and release of adrenaline was probably not of sufficient magnitude to deplete the adrenals of adrenaline. This explanation seems especially logical since the resynthesis of adrenaline is normally quite rapid (23–25).

It would then follow that if the demand (release) were exceptionally great, the adrenals could be partially or totally depleted of their adrenaline storage. Such a condition has been demonstrated experimentally by administering insulin (1) and acetylcholine (3). In such a situation where demand (release) is especially great, the urinary output of adrenaline would remain elevated only as long as the reserve supply was intact. Once the storage of adrenaline is depleted, the urinary output of adrenaline would be governed by the rate of adrenaline resynthesis. Since resynthesis is normally rapid (23–25), one would expect a fairly high urinary output of adrenaline. Patients in Group B, Table I, showing a high urinary output of adrenaline and low content of adrenaline in the adrenal gland, would apparently represent such a situation.

The third group (Group C) of patients, which represents 71.5 per cent of all the burned patients who died, showed a low urinary adrenaline and low adrenaline content of the adrenal gland (Table II). In general, these patients at first were capable of producing large quantities of adrenaline, as is seen by their initial high output. However, the adrenal medulla was apparently unable to synthesize adrenaline rapidly enough and certainly not at a rate commensurate with demand; consequently, the adrenal gland was largely depleted of its adrenaline, leading to a terminal picture of low urinary adrenaline and a low adrenaline content of the adrenal gland; that is, an acute medullary insufficiency developed.

Why an acute adrenal medullary insufficiency develops is difficult to say. However, it would appear that this insufficiency is typical to these particular burn patients and represents an inherent failure on the part of their medulla. This seems especially plausible since the normal adrenal medullary tissue can synthesize large quantities of adrenaline for long periods of time, as is characteristically seen in severely burned patients who survive (7). Although the normal medulla rapidly synthesizes adrenaline, the extent of this synthesis is at least in part dependent upon the inflow of the necessary adrenaline precursors. Since the biosynthetic pathway for adrenaline formation is through phenylalanine, tyrosine, dopa, hydroxytyramine and noradrenaline (28–30) (Figure 1) and since the adrenal medulla can synthesize adrenaline from tyrosine (28), dopa (28), hydroxytyramine (28), or noradrenaline (31), a deficiency in any one of these precursors could ultimately lead to a deficit in adrenaline formation. It is further known that hepatic damage commonly occurs in severely burned patients and, since the metabolism of phenylalanine (32, 33) and tyrosine (34, 35) is so closely linked with liver function, it would then seem logical that hepatic failure might indirectly also play a part in the failure of the adrenal medulla to synthesize adrenaline. Finally, after prolonged periods of infusion which often occur in treating burned patients, an inade-

quate diet itself might then possibly play a contributory role in the adrenaline deficit. Studies are currently under way to determine the therapeutic use of various adrenaline precursors in severe burns.

In evaluating the etiology of adrenal medullary insufficiency following thermal burn, one must also consider the possible role of the sympathetic nervous system in adrenaline synthesis. Since the adrenal medulla in the absence of sympathetic innervation readily synthesizes adrenaline (28), it would appear that the sympathetic nerves, per se, have little or no direct control over adrenaline synthesis. Therefore, the premise that the medullary failure might originate in the sympathetic nervous system or higher centers seems unreasonable. On the other hand, the sympathetic nerves to the adrenal medulla are intimately related to the release of adrenaline (6, 36).

To use a very crude analogy as a means of recapitulation, the adrenal medulla resembles a reservoir in which the inflow is represented by resynthesis of adrenaline and the outflow by the demand for and release of adrenaline. If the demand (release) exceeds the rate of resynthesis, the reservoir is depleted and the output is then governed entirely by resynthesis. If the resynthesis is rapid, then the demand is fulfilled and the reservoir remains full. In some patients the inflow, or rate of resynthesis, is apparently inadequate to meet the heavy demands imposed by the severe "stress" of burn and, consequently, the adrenals are emptied of their adrenaline storage. This latter picture would, therefore, represent an acute medullary insufficiency since the failure is ultimately in the rate of resynthesis. In view of adrenaline's important metabolic (37–40), cardiac (38, 41–43), and vascular action (44–46), this insufficiency is more than likely an important contributory factor in the death of the patient.

Although this paper has devoted its attention strictly to acute adrenal medullary insufficiency in severe thermal burns, it would certainly seem that a similar picture could develop in other severely stressful situations such as those which might be seen after extensive surgery, severe trauma, necrotizing pancreatitis, extensive myocardial infarction, meningococcal septicemia and so forth, and, as has been recently shown, in X-irradiation (47).

SUMMARY

1. The urine and adrenals of 14 burned patients who died were assayed for adrenaline and noradrenaline.

2. Approximately two-thirds of these burned patients showed at death a subnormal adrenaline output and a low adrenaline content of the adrenal gland, a condition commensurate with acute adrenal medullary insufficiency.

3. The possible mechanism of this adrenal medullary insufficiency was discussed.

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STRUCTURAL UNITS OF HUMAN 7S GAMMA GLOBULIN *

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A number of observers in the past (1–3) have successfully degraded rabbit and horse antibodies with various enzymes, thus producing fragments retaining antibody activity. More recently Porter (4, 5) has produced split products of rabbit γ-globulin by treatment with papain and cysteine, two of which retained antibody activity as measured by their ability to inhibit precipitation of the original antiserum with homologous antigen, while the third appeared to retain the greatest antigenic specificity. Karush (6) and Nisonoff, Woernley and Wissler (7, 8) working with antihapten antibodies have clearly shown that these fragments are univalent. Putnam, Hsiao and Tan (9), employing similar techniques, have been able to split human γ-globulin and myeloma proteins into two major chromatographically separable fractions with sedimentation coefficients of approximately 3.5S. A preliminary report of some physical, chemical and immunological properties of similar subunits from human γ-globulins and several human antibodies has been presented (10). In the present report, results are presented which indicate that following digestion of human γ-globulin there are produced at least three fragments differing from each other in chemical properties. The major part of the antibody activity against three different antigens was found to be associated with one of these fragments, while smaller amounts were present in a second, antigenically similar fragment. The third fragment contained virtually no antibody activity but had additional antigenic specificity.

MATERIALS AND METHODS

Human γ-globulin was obtained as Cohn fraction II (Lederle, Lot no. C576). Ultracentrifugal examination demonstrated that this preparation consisted of approximately 95 per cent 7S γ-globulin, and that it contained less than 5 per cent of molecules with s rates of 9 to 11S and no detectable amounts of 19S γ-globulin. For studies of specific antibodies, γ-globulins were also isolated by starch zone electrophoresis (11) from the serum of a patient who developed serum sickness following the injection of tetanus antitoxin, and by precipitation with 34 per cent saturated ammonium sulphate from the serum of a patient hyperimmunized with diphtheria toxoid.

Breakdown of γ-globulin was carried out as described by Porter (5) in pH 7.0 phosphate buffered saline, 0.01 M cysteine and 0.002 M ethylenediamine tetraacetate (EDTA) (Versene), using 1 mg twice recrystallized papain per 100 mg protein.[1] The breakdown was allowed to proceed at 37° C for 12 to 18 hours. The material was then dialyzed for 4 hours against 50 to 100 ml pH 7.6, 0.01 M phosphate buffer and then against 1 L of the same buffer for 24 hours. The amount of dialyzable material was determined by the modified Folin technique using γ-globulin as a standard (12). The exact nature of these products was not examined further. In a number of experiments, 3 mg pepsin per 100 mg γ-globulin at pH 4 was used, alone or in conjunction with subsequent treatment, with 0.1 M mercaptoethanol as described by Nisonoff, Wissler and Woernley (8).

Ultracentrifugal analyses. Ultracentrifugal analyses were carried out in a Spinco Model E ultracentrifuge, using cells with a double sector centerpiece. Quantitative measurements were carried out directly from the photographic plates using a comparator (Gaertner) according to methods described by Trautman (13). Preparative ultracentrifugation was performed in a Spinco Model L ultracentrifuge, using a swinging bucket rotor (SW-39) and a saline gradient as described previously (14).

Chromatography. Chromatography was carried out on carboxymethylcellulose (CM cellulose) or Diethylaminoethylcellulose (DEAE cellulose) as described by Peterson and Sober (15).[2] For preparative purposes glass columns, 2.5 cm OD and 40 cm long, were employed. Ten or 20 g of adsorbent was washed with distilled water and the starting buffer and packed into the column with approximately 10 pounds per square inch air pressure to a height of 15 and 30 cm, respectively. The columns were equilibrated with a continuous flow of 2,000 ml starting buffer. Two hundred fifty to 500 mg of γ-globulin was dialyzed for 4 to 24 hours against the same buffer and carefully applied to the column. In the initial experiments, elution was carried out by gradient elution at con-

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[1] Prepared by Dr. J. Potter.

[2] Purchased from Brown & Co., New Hampshire: DEAE cellulose, Lot no. 1078, 1066, capacity 1 mEq per g; CM cellulose, Lot no. 1100, capacity 0.7 mEq per g.

stant pH and increasing ionic strength, as described by Fahey and Horbett (16). The gradient was started after collection of the protein that came off with the solvent front. Since only a single additional peak was obtained from each column, subsequent studies were carried out by stepwise elution. Elution from the cation exchanger was carried out with pH 7.6 phosphate buffer and from the anion exchanger at pH 8.0. Ionic strengths of 0.4 and 0.3, respectively, were obtained by adding sodium chloride to 0.01 M phosphate buffer. Ten-ml aliquots were collected with an automatic fraction collector, and protein was measured by reading the extinction in a Beckman DU spectrophotometer at 280 mμ. An approximation of the amount of protein in each tube was obtained by comparison with a standard curve prepared with Cohn fraction II γ-globulins. These values can be considered only as an approximation, since the amino acid composition of the fragments is not known.

Immunological and chemical studies. Immunological studies were carried out using a pooled antiserum to 7S γ-globulin prepared in five rabbits over a prolonged period of immunization, and antisera to the whole unfractionated digested γ-globulin prepared in rabbits with Freund's adjuvant as previously described (17). Studies were carried out by double diffusion in agar by the technique of Ouchterlony (17), and by micro-immunoelectrophoresis by the technique of Scheidegger and Buzzi (18). Quantitative precipitin analyses were done as described previously (17) using 0.05 to 0.5 ml antiserum.

Hexose was determined by the anthrone reaction (19) using a mixture of glucose, fucose and mannose as a standard, and hexosamine by a modification of the Elson-Morgan reaction using glucosamine as a standard (20). To calculate the hexose protein ratio of the fractions, protein was measured by the modified Folin technique using Cohn fraction II γ-globulin as a standard (12).

Antibody assays. 1) Precipitating antibody to horse serum proteins in electrophoretically isolated γ-globulins from a patient with serum sickness was assayed by quantitative precipitin techniques (17). Inhibitory power of digested fragments was determined by quantitating their ability to decrease the amount of precipitate between the original antiserum and homologous antigen, and by their capacity to inhibit the passive cutaneous anaphylactic reaction when mixed with the antiserum injected into the skin of the guinea pig (21).[3]

2) Antibodies to diphtheria toxin in γ-globulins isolated from a hyperimmunized normal subject and in fragments prepared from them, were measured by their capacity to neutralize the effect of the toxin in the skin of a rabbit (22).[4]

3) Antibody to streptokinase was present in the preparation of Cohn fraction II, Lot no. 576, employed in these studies and was estimated by its ability to inhibit the activity of streptokinase using the fibrinolytic assay described by Johnson and Tillett (23).[5]

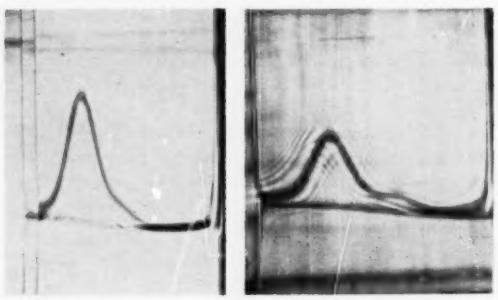


FIG. 1. ULTRACENTRIFUGAL PATTERNS OF TWO PREPARATIONS OF DIGESTED γ-GLOBULIN SHOWING IN ADDITION TO THE MAJOR 3.5S PEAK, THE PRESENCE OF VARIABLE AMOUNTS OF A MORE RAPIDLY SEDIMENTING 5 TO 6S COMPONENT; SPEED 52,640 RPM. Photographs taken after 64 minutes. Sedimentation proceeds from left to right.

RESULTS

Treatment of 7S γ-globulin with papain, cysteine and EDTA, as described under Methods, resulted in virtually complete conversion of the starting material which had an observed s rate of 6.5 to 6.8S into a major fraction with sedimentation coefficient of 3.4 to 3.5S and small but variable amounts of more rapidly sedimenting material with a sedimentation coefficient of 5 to 6S. This sedimented somewhat more slowly than the starting material and probably represents partially degraded γ-globulin. Approximately 80 to 90 per cent of the total was not dialyzable, while the remaining 10 to 20 per cent appeared as dialyzable nitrogenous products. Two preparations of treated γ-globulin prepared from the same lot but showing different amounts of the more rapidly sedimenting material are shown in Figure 1.

When the digest was subjected to chromatography on a CM cellulose column at pH 7.6 using a gradient of 0.01 to 0.4 M NaCl, two major peaks were recovered. One of these came off with the starting buffer, and the second one shortly after the start of the gradient. Since this second peak could not be resolved further, subsequent experiments were carried out by stepwise elution using the starting buffer and 0.4 M NaCl, pH 7.6. The results of one such experiment are shown in Figure 2 (top). The amounts of protein associated with each peak were quite variable; in general, peak I contained somewhat less protein than peak

[3] Kindly performed by Dr. Zoltan Ovary.
[4] Kindly performed by Dr. Jonathan Uhr.
[5] Kindly performed by Dr. Alan Johnson.

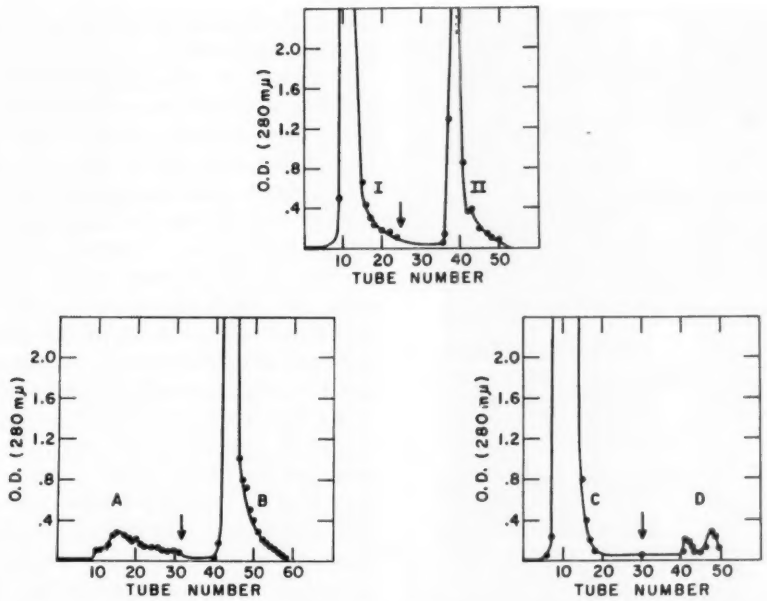
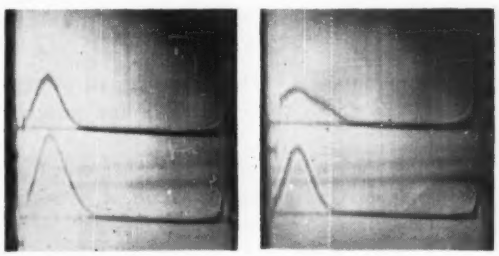


FIG. 2. CHROMATOGRAPHY OF DIGEST OF HUMAN γ-GLOBULIN. Top: first step on CM cellulose, pH 7.6. Arrow indicates change of eluting buffer. Bottom: rechromatography of peak I (left) and peak II (right) on DEAE cellulose, pH 8.0. Arrow indicates change of eluting buffer.

II. In some experiments, however, the reverse occurred. Rechromatography of each of the peaks revealed peak I to be quite pure. However, peak II frequently contained small amounts of contaminating protein and was therefore rechromatographed in a number of experiments before further separation was undertaken. While this step appeared to have resulted in appreciable resolution, immunological and chemical studies to be described below indicated that each fraction was still heterogeneous. Each fraction was therefore chromatographed further on a DEAE cellulose column at pH 8.0, 0.01 to 0.3 μ. By gradient elution each fraction could be further resolved into a major and one minor peak (IA and B) (IIC and D). Since in each instance only a single peak was recovered from the DEAE cellulose column after the starting buffer, subsequent experiments were again performed by stepwise elution with similar results. The peaks obtained in one such experiment are shown in Figure 2 (bottom). Rechromatography of each peak under identical conditions revealed only a single peak in the same position, thus indicating that these represent different molecular species and not simply artifacts created during the fractionation procedure. The relative amounts of each fraction were variable. In general, fraction C was the largest single component and made up from 40 to 50 per cent of the total protein, while fraction A made up from 15 to 25 per cent of the total protein recovered. About one-quarter to one-third was recovered with fraction B. Fraction D rarely contained more than 5



1 2

FIG. 3. ULTRACENTRIFUGAL PATTERNS. 1: Fraction A (top) and fraction B (bottom). 2: Fraction D (top) and fraction C (bottom). Speed 42,040 rpm. Photographs taken after 64 minutes. Sedimentation proceeds from left to right.

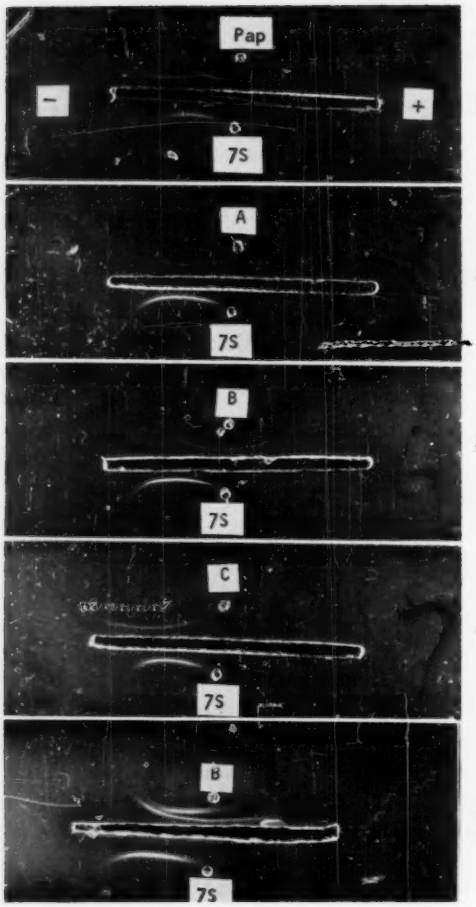


FIG. 4. IMMUNOELECTROPHORETIC PATTERNS COMPARING 7S γ-GLOBULIN ON THE BOTTOM OF EACH PATTERN WITH: 1) WHOLE PAPAIN DIGEST; 2) FRACTION A; 3) FRACTION B, 4) FRACTION C; AND 5) A SECOND PREPARATION OF FRACTION B. Anode is to the right.

per cent of the total. Total recovery of protein ranged from 85 to 95 per cent of the material applied.

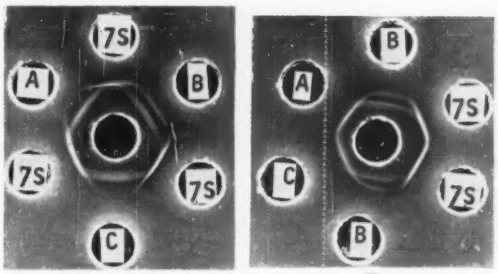
Examination in the analytical ultracentrifuge demonstrated A, B and C to sediment as single rather homogeneous peaks with an observed s rate of 3.4 to 3.5S (Figure 3). D was heterogeneous and consisted of approximately equal amounts of two peaks with s rates of 3.5S and 5 to 6S (Figure 3). Molecular weights calculated by the Archibald technique were obtained for A, B and C and ranged from 40,000 to 55,000 for each. Fraction D was too heterogeneous for such analyses and was not examined further.

In view of the well recognized heterogeneity of γ-globulins and the lack of molar equivalence between these fractions, it was of interest to determine if they were derived from different fractions of γ-globulin. Chromatography of whole Cohn fraction II γ-globulin on CM cellulose at pH 7.6 results in two major fractions. One of these is eluted with the starting buffer, is slightly less basic, and has a slightly more rapid electrophoretic mobility than the second one, which is eluted at higher salt concentration. When each of these fractions was separately digested with papain and chromatographed in a manner similar to that used for whole γ-globulin, both fractions yielded approximately equal amounts of fraction B. However, they differed in the relative amounts of the two other peaks. The fraction eluted first yielded large amounts of fraction A and little C, while the second one yielded larger amounts of fraction C and only small amounts of A. Small amounts of fraction D were obtained from both. Similarly when γ-globulin was fractionated into a slow moving and rapidly moving fraction by starch zone electrophoresis prior to digestion, relatively larger amounts of fraction C were isolated from the former, while the more rapidly migrating protein contained predominantly fraction A. The relative amount of fraction B was approximately the same in both.

Antigenic properties. Figure 4 (top) compares the immunoelectrophoretic patterns obtained with 7S γ-globulin and the whole papain digest when they react with rabbit antiserum to 7S γ-globulin. While the native proteins gave only a single line, the papain digests formed two major lines which appeared to be independent of each other. One of these migrated more rapidly and the other more slowly than γ-globulin. Occasionally, a third line was also visible near the point of application. The four lower patterns in Figure 4 show similar studies with each of the separated fragments. Fragment C corresponds to the major slow line of the whole digest, B to the major fast one, and A has an intermediate mobility but could not always be detected in the whole digest.

In a number of experiments fraction B had a second line near the origin (Figure 4, bottom). Fraction D was heterogeneous and contained each

of the lines present in the whole digest and was therefore not studied further. While fractions A and C differed from each other in mobility, no distinct differences could be detected between them by double diffusion according to the method of Ouchterlony. Figure 5 compares the reaction of each fraction and 7S γ-globulin with a rabbit antiserum to 7S γ-globulin. It can be seen that the single major lines produced by fractions A and C fused with each other to give a reaction of identity (Figure 5B) but were antigenically deficient when compared to the native protein (Figure 5A). Occasionally a second faint outer line was visible (Figure 5A) which probably represented traces of contaminating material. Fraction B contained two major components which together appeared to have all of the constituents of the native protein (Figure 5A). Figure 5B demonstrates that one of the lines in fraction B is antigenically distinct from A and C and crossed through them, while the second one appeared to be similar to them. Both lines in fraction B appear to be of the 3.5S class and not due to undegraded 7S γ-globulin, since they were recovered in the top three fractions obtained by density gradient ultracentrifugation. Only a small portion of 7S γ-globulin, simultaneously separated in another tube, was found at this level.



A B

FIG. 5. OUCHTERLONY DOUBLE DIFFUSION PLATES COMPARING THE FRAGMENTS: A) WITH 7S γ-GLOBULIN; B) WITH EACH OTHER, USING AN ANTISERUM TO 7S γ-GLOBULIN IN THE CENTER WELL.

Differences between these fractions and native 7S γ-globulin are also demonstrated by precipitin analyses shown in Figure 6. None of the frag-

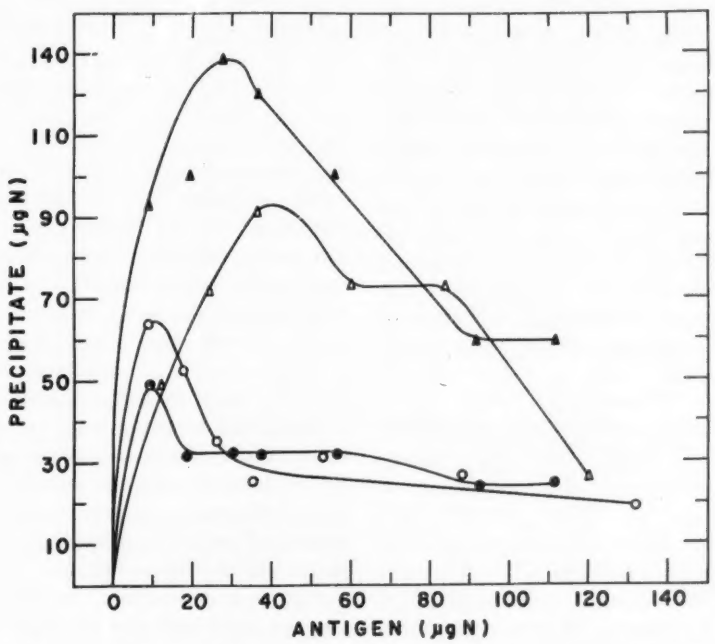


FIG. 6. PRECIPITIN ANALYSES COMPARING THE REACTIONS OF FRACTION A (○), B (△), C (●) AND 7S γ-GLOBULIN (▲) WITH AN ANTISERUM TO 7S γ-GLOBULIN.

TABLE I

The effect of the papain-digested fragments from antibodies to horse serum proteins on the precipitation of horse serum by the untreated antiserum

Fraction		Horse serum-human antiserum	Human albumin-rabbit antiserum
	mg	*μg ppt*	*μg ppt*
None		88.0	475
Papain digest	1.28	14.0	480
A	1.25	42.5	475
C	1.36	10.5	475
D	0.50	57.0	*

* Not determined.

ments precipitated as much antibody as did the original antigen. Supernatant analyses with 7S γ-globulins in antigen excess revealed complete neutralization of antibody by 7S γ-globulin, but residual activity against 7S γ-globulin remained in the region of antigen excess of fractions A and C.

Preliminary studies with antisera to the whole papain digest offered less resolution and little additional information.

Antibody analyses. Antibody activity of three human antibodies was tested following breakdown. Only one, an antiserum to horse serum, was a precipitating antibody under the conditions of the study. Following breakdown to 3.5S fractions this antibody no longer precipitated with antigen, nor did any of the separated fragments. However, the whole papain digest inhibited the precipitation of the original antiserum and of the homologous antigen almost completely (Table I). When each of the isolated fractions was tested, fraction C had most of the inhibitory activity, while A was less active at the same concentration. This particular experiment yielded amounts of fraction B too small for assay. Table I also indicates that none of the fractions had any effect on a heterologous system of human albumin and a rabbit antiserum. Similar results to be reported in detail separately were obtained by studying the inhibition by these fractions of passive cutaneous anaphylaxis, employing the antiserum and horse serum (24). Here, too, fraction C and the whole papain digest were active. Fraction D did not inhibit, while fraction A had a nonspecific hemorrhagic and inhibitory effect on the homologous as well as on the heterologous antigen-antibody reaction.

Activity of the fractions produced from the other two antibodies was measured by their ability to neutralize toxin. In the case of diphtheria antitoxin, the rabbit skin assay was employed (22); for antibody to streptokinase the kinase inhibition was measured (23). Only 15 mg of antidiphtheria γ-globulin was available and fractionation was not carried out beyond the first step of chromatography on CM cellulose. The results of these studies are listed in Table II. In each case the papain digest retained virtually all of the original activity. Following fractionation the bulk of activity was associated with the second peak eluted from the cation exchanger (peak II) in the case of diphtheria antitoxin, and with fraction C for streptokinase. In each instance, as in the case of the antibody to horse serum, about 15 to 25 per cent of the total activity was also associated with fractions I and A, respectively.[6] The significance of this will be discussed below.

TABLE II

Antibody activity of papain-digested fragments prepared from diphtheria antitoxin and antistreptokinase

Fraction		Diphtheria antitoxin	Antistreptokinase
		U/mg	*U/mg*
γ-Globulin		10	2.7
Papain digest		10	2.8
A	I	1.3	2.7
B			0.2
C	II	6.7	8.2
D			0

TABLE III

Carbohydrate composition of papain-digested fragments of Cohn fraction II γ-globulin

	Per cent hexose		Per cent hexosamine	
γ-Globulin	1.2		1.0	
Papain digest	1.2		1.1	
Fraction	Prep. 1	Prep. 2	Prep. 1	Prep. 2
A	1.0	1.4	0.7	0.6
B	3.0	3.1	1.7	2.0
C	0.2	0.3	0.3	0.4

Carbohydrate analyses. In view of previous reports on the isolation of small glycopeptides from

[6] Qualitatively similar results have recently been obtained with another precipitating antibody than that to thyroglobulin.

γ-globulin (25), it seemed of interest to determine the carbohydrate content of fractions prepared from Cohn fraction II. Table III indicates that digestion did not result in significant loss of carbohydrate. Following chromatography, fraction II and subfractions C and D contained virtually no hexose or hexosamine; the bulk of the carbohydrate was associated with fraction I. Although results were variable, probably because of the presence of small degrees of impurities in each fraction, fraction B contained the largest amount of carbohydrate and approximately 3 per cent hexose and 2 per cent hexosamine, while A had somewhat lower values. The results of two representative experiments are listed in Table III. The first preparation represents fractions that had been rechromatographed on DEAE cellulose prior to analysis; the second preparation had been refractionated on CM cellulose. Thus, more than 75 per cent of the total hexose and hexosamine recovered in this preparation was associated with fraction B.

DISCUSSION

Normal human 7S γ-globulin has a molecular weight of approximately 160,000 and has been shown to consist of at least two peptide chains on the basis of careful end group analyses (26, 27). Detailed structural studies have been hampered not only by the large size and complex structure of the γ-globulin molecules, but also by the heterogeneity of γ-globulin (which should be called γ-globulins). Similar difficulties have also been encountered in the study of antibodies produced in other species. Thus, the basic question, whether antibodies to different antigens differ in their amino acid composition and primary structure, or whether antibody specificity is due to changes in the secondary structure of preformed γ-globulin molecules resulting from contact with the antigen, has not been answered. Previous studies, especially those of Kabat (28), have indicated, however, that the antibody combining sites are small, and carbohydrate analyses by Rosevear and Smith (25) indicate that the carbohydrate is associated with certain specific areas of the molecule. The recent observation of Porter (5) that rabbit γ-globulin can be reproducibly broken down into easily separable fragments with molecular weights of 50,000 to 80,000 suggested that similar studies of human γ-globulins and antibodies might provide some answers to these basic questions and facilitate further structural analyses of the γ-globulin molecule.

The results of the present study indicate that human γ-globulin can also be broken down into fragments having one-fourth to one-third the size of the original molecule. However, careful analyses of some of these units reveal certain differences which suggest a structure for human γ-globulin which differs from that of rabbit γ-globulin. The fragments so produced can be separated by chromatography into three major components differing in chemical and electrophoretic properties and, to some extent, antigenically. The fragment with the most rapid electrophoretic mobility and an additional antigenic component did not contain significant antibody activity, but contained more than three-quarters of the carbohydrate present in the original molecule. Most of the antibody activity was associated with the fraction with the least negative charge on immunoelectrophoresis (fraction C) which contained little carbohydrate; however, significant antibody activity was also associated with the fragment that was antigenically closely related to the latter (fraction A) but contained more carbohydrate and had a somewhat greater negative charge. The presence in γ-globulins of an antigenically distinct F component and of several antigenically closely related S and mid-components, which contribute to the electrophoretic mobility of the γ-globulins, has also recently been described by Edelman, Heremans, Heremans and Kunkel (29) in a study of the double line phenomenon. Studies with Cohn fraction II γ-globulin containing antibody to streptokinase indicate that it consists of two major fractions which can be separated by chromatography. These fractions differ somewhat in electrophoretic mobility and carbohydrate content, but resemble each other in having approximately similar amounts of antibody activity to streptokinase. Both fractions contain a common non-antibody fragment (fraction B) which is rich in carbohydrate and makes up about one-fourth to one-third of the molecule. Some of their differences appear to be due to the presence of larger amounts of the more basic fragment C in the γ-globulin with the least negative charge and slower electrophoretic mobility, while greater amounts of the

more rapidly migrating fragment A were recovered following digestion of the fraction of native γ-globulin with the higher electrophoretic mobility. It would thus appear that the electrophoretic mobility of the native protein is partially determined by the relative amounts of those fragments which also have the antibody combining sites.

The chemical analyses are consistent with previous reports on the association of carbohydrate with certain low molecular weight peptides with molecular weights of less than 5,000 (25). Of particular interest in these studies is the demonstration of the existence of at least two distinct antigenic fragments. While comparison of these with native γ-globulin suggests neither loss nor release of antigenic groups present in the native molecule, preliminary studies, comparing them to fragments produced by treatment with pepsin and mercaptoethanol as described by Nisonoff and associates (8), suggest the possible loss of a few antigenic groups present in the native protein following digestion with papain.

The exact nature of the bonds holding the fragments together cannot be determined with certainty from these studies. However, the observations that a number of closely related smaller fragments can be prepared from rabbit and human γ-globulins by sulfhydryl reagents combined with denaturing agents (30), papain (4, 5) or pepsin (8) suggest that the common mechanism may be the reduction of disulfide bonds by the sulfhydryl reagent, and that the enzyme or denaturing agent may make these accessible by unfolding the molecule or by breaking off a fragment. While the close similarity of the antigenic properties of fractions A and C when tested with rabbit antisera raises the possibility that they are identical, such a view does not appear likely, since they differ in some of their chemical properties, in electrophoretic mobility, and in antigenicity when tested with antisera produced in different species (31). Furthermore, the differences in the composition of γ-globulins fractionated by several techniques prior to digestion, suggest that γ-globulins consist of a heterogeneous group of proteins differing in the relative amounts of these fractions. It is of interest that a small fraction (about 5 per cent) of the γ-globulin resists breakdown to the 3.5S units.

The production of 3.5S units and of nonprecipitating fragments is not only of theoretical interest in elucidating the structure of γ-globulin, but may eventually shed light on the synthesis and specificity of antibody structure. In addition, such nonprecipitating fragments with antibody activity may prove of use in neutralizing certain noxious antigens without the danger of producing tissue damage caused by the interaction of antigen with antibody fixed to tissues. Ovary and Karush (21) have shown that antibody-containing fragments of rabbit γ-globulins do not fix to the skin and, therefore, presumably not to other tissues. Similarly, none of the human fragments appears to fix to the skin as measured by reverse passive cutaneous anaphylaxis (31). This observation would suggest that such univalent fragments may be of use in neutralizing circulating noxious antigens without the production of tissue damage before they can elicit an antibody response by the host. Similarly, the role of these fragments in the fixation of complement and other immune mechanisms, and the relationship of these artificial fragments to those produced *in vivo* (32) remain to be explored.

SUMMARY

1. Breakdown of human 7S γ-globulin with papain and cysteine results in three major chromatographically separable fractions with sedimentation coefficients of 3.4 to 3.5S, molecular weights of 40,000 to 55,000, and small amounts of partially degraded γ-globulin.

2. Each of these is antigenically related to the native protein. Two of these (A and C) are closely related to each other but differ in electrophoretic mobility, while the third one (B) differs from them also antigenically.

3. Most of the carbohydrate is associated with fraction B; more than three-fourths of the antibody activity against three different antigens is found in fraction C, with small amounts in fraction A.

4. The role of these structural units in determining some of the known properties of γ-globulins is discussed.

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ENZYMES AND BLOOD CLOTTING. I. TRYPSIN AS AN ACCESSORY FACTOR *

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There is considerable literature, cited (1), on various agents which, besides having proteolytic activity, can influence blood clotting. Some of these are crystalline enzymes, such as trypsin and papain. Others are crude materials, in which the active principle has yet to be identified. The ways in which clotting is affected by these diverse agents have been studied to varying extents. There have also been relatively recent discoveries of a number of *natural* "accessory" factors, participating in the thromboplastin- and thrombin-forming reactions. These will be referred to by the Roman numeral designations of the International Committee on Blood Clotting factors (2), or by one or two of the common abbreviated synonyms, as follows: V (AcG), VII [SPCA, or "proconvertin" (ProC.) in the strict sense], VIII (AHF), IX (PTC, or Christmas factor), X (Stuart-? Prower), PTA (plasma thromboplastin antecedent), Hag (Hageman factor). The time is especially ripe to work out what relationships may exist between these accessory factors and the "enzymes" in question.

Illustrating some of the agents which will be included in the present series of investigations, Table I shows a heterogenous group of additives all of which can clearly accelerate the clotting of whole blood (in silicone). The following methods of study will be followed. First, the agents will be added to a variety of routine nonspecific clotting test systems. Second, they will be tested in one-stage (3) and two-stage (4) systems, which have been devised for the specific assay of individual clotting factors. By testing in systems deficient in just one factor at a time, it is possible to bring out relationships between the test agents and the natural clotting factors. Finally, the indications afforded by the preceding results will be followed up with special tests in order to define more precisely the modes of action of each agent studied. Where possible, effects of "combining" each clotting factor with the enzyme preparation will be tested on appropriate clotting systems.

In the first paper of the series, these considerations will be illustrated by an experimental analysis of the effects of trypsin. The ability of trypsin to accelerate blood clotting was discovered by Douglas and Colebrook (5) in 1916; in 1937, Eagle and Harris (6) gave evidence for its role in the activation of prothrombin to thrombin. We (1) have contributed many publications in the past 20 years (1939 to 1959) to explain the action of trypsin in the thrombin-forming system as that of a "thromboplastic enzyme" (7) (see Discussion), working in conjunction with Ca ions and a prothromboplastic lipid (4), which can be provided by platelets, cephalin and so forth. Many of our earlier experiments, however, preceded the era of modern knowledge of the natural accessory factors and it is particularly in relationships to these that the present data bring out some important new concepts.

FOREWORD ON CLOTTING TEST METHODS

Although most readers will be familiar with the routine clotting test procedures, with the possible exception of some of the "specific" methods, there are many facts and ideas implicit in their interpretation which are not widely appreciated.

Nonspecific tests. Since blood does not clot while normally circulating *in vivo*, any clotting must be the result of nonphysiological circumstances. Many factors are now known to participate in the reactions of blood coagulation. We are, for the most part, still far from the goal of identifying and quantitating them biochemically. Almost all progress in this field relies upon tests of "activities," whic. determine clotting times in vari-

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ous multicomponent test systems, affected by numerous variables.

In clotting tests performed on shed (e.g., arm-vein) blood, much depends upon the technical details of handling. Avoidance of tissue contact, for instance, is generally emphasized. In the "spontaneous" clotting of such whole blood, *in vitro* at body (37° C) temperature, the chief variable is the contact surface(s). It is now a common routine to test in both glass and silicone. There is good recent evidence (8, 9) that the PTA and Hageman factors interact in the presence of certain surfaces, e.g., glass, to form a powerful accelerator of the initial phases of blood clotting. "Surface factor" (SF) (10) or "activation product" (11) are convenient terms for this.

In plasma recalcification (PCa) tests, factors connected with the technical handling must be expected to afford opportunity for formation of this SF. Plasma is collected, with varying technical skill, by receiving blood into some anticoagulant, usually citrate or oxalate, centrifuging (another variable), storing and so forth, under various conditions of exposure to surfaces, temperature changes and the like. Traces (or more) of the formed elements, especially platelets, are not negligible contaminants. Only a few workers, including ourselves, have obtained plasmas so free from platelet material as to be incoagulable on simple recalcification (1). Our ordinary routine is to use the silicone technique, merely centrifuging the (citrated) blood in the cold (4° C) for 30 minutes at 3,000 rpm, to obtain what we, hopefully, call "platelet-poor" plasma. Typically, this gives PCa times of 5 to 7 minutes soon after preparation, but this is reduced to 2 to 3 minutes in a few hours, especially on exposure to glass surfaces and room temperatures. Evidently, the benefits of platelet removal are partly counteracted by plasma "activation" phenomena. We probably do not know all possible activations which may occur in shed blood and plasma.

On the other hand, there are also "removals." Depending largely on storage conditions, some clotting factors are labile. There are species variations. In human blood or plasma, factor V (AcG) is particularly labile and factor VIII (AHF) moderately so. Freezing and thawing plasmas, or preparing them in the lyophile-dried state, are also productive of alterations. Even a trace of clotting can affect a blood sample considerably. Serum has its own problems as to clotting factor components, and so do all fractions. Sometimes plasmin (fibrinolysin) complicates the picture. The over-all practical question concerns the quantitative level of each clotting factor present and its ability to affect the results of the clotting test in question. We are very apt to take for granted the nonvariance of many factors when this is merely fortuitous under most ordinary circumstances. There is actually a dearth of accurate information on many of these technical points.

The PT (prothrombin time) test of Quick (12), is performed in our laboratories by adding to 0.1 ml oxalated (or citrated) plasma, 0.1 ml of optimal strength human brain thromboplastin, and 0.1 ml 0.02 M $CaCl_2$, at 37° C. Our normal human plasmas, routinely tested in glass after a few hours from the blood collection, usually yield a PT time of 11 to 12 seconds. The Quick test clotting times are so short that it is difficult to assess the significance of variations less than 1 to 2 seconds. Valuable as this test is, it can detect only relatively gross factor variations. PT's are significantly lengthened in deficiencies of factors II (prothrombin), V (AcG), VII (SPCA) and X (Stuart) but, rarely, because of very low fibrinogen (I), or in the presence of heparin and some other inhibitors. While properly including the Quick test in the nonspecific group, it is, nevertheless, recognized for its value as a screening test for the limited group of factors mentioned above.

The PTT (partial thromboplastin time) test, developed by Langdell, Wagner and Brinkhous (13), we now perform routinely with optimal (usually 0.06 per cent) human brain (crude) cephalin (14) substituted for the thromboplastin of the PT test. The PTT test is sensitive to variations in, or additions of, V, VIII, IX, X, PTA, Hageman, and SF. Surface factor may be formed in glass or added in certain reagents (10). As with the PCa tests, and probably for the same reasons, the normal PTT controls show considerable variation. A good routine is to keep the platelet-poor plasma in an ice-bath and test as soon as possible. Of course, this is impracticable with many plasmas, especially those from clinical patients. When saved for "substrates" (see below), most of these are kept frozen-stored at $-20°$ C in siliconed or lusteroid tubes. Occasionally, we have available only lyophilized substrates, but the test results on these are regarded with considerable reserve.

Specific one-stage tests. When a substrate plasma is chosen, which is specifically deficient in the factor to be tested, prolonged clotting times will be obtained in one or more of the above nonspecific tests. If the missing factor is added to the substrate, the clotting time will be reduced, and this can be made the basis for a bioassay. Equal 0.1 (or 0.05) ml volumes of substrate plasma and the unkown are mixed and tested with the appropriate additions of calcium and thromboplastic agent. The clotting times may be compared by inspection or quantitated as activities by reference to a series of standards. Details are given in previous publications (1, 3). A 1:10 dilution of (standard) normal human plasma is conveniently regarded as 100 per cent (of normal), especially in testing unknown plasmas similarly diluted. Specific PT-type tests are set up in this way for factors II (prothrombin), V (AcG), VII (SPCA) or X (Stuart) *separately*, or (VII + X) *together* [after Owren (15)].

Similarly, the PTT-test principle is applied specifically by adding the test material to substrate plasmas respectively deficient in V, VIII (AHF), IX (PTC), X, PTA, or Hageman. Variables which are apt to be overlooked may include: *1*) uncontrolled SF formation (see above); *2*) loss of V; *3*) variation of more than one factor at a time; *4*) special inhibitor problems, which are rare (16) [which may also affect PT tests and so forth (17)]. It

must be remembered that each one of the cited clotting factors makes its own quantitative contribution to the clotting test and that all are necessary.

Two-stage tests. The principle of the two-stage test is: first, to incubate together the factors necessary to form thrombin; and second, to follow the thrombin formation by adding successive samples of incubate to a standard test fibrinogen. Other things being equal, the more thrombin formed, the shorter the clotting time. A plot of the data yields a "prothrombin activation curve (18) affording information on both rate and yield. We always follow through the endpoint (optimum) or shortest clotting time reached, and report this together with the incubation period (opt.) needed, as well as the initial (0.5 or 1 minute) data. Intervening values provide little additional information. Conditions for these tests must be rigorously standardized. This is most satisfactorily achieved in the research laboratory, but, like any other special procedure, can be learned through experience. Our particular type of two-stage test is the oldest, dating back to the original discovery of prothrombin and of the roles of calcium and tissue extracts. Its special features are: *1*) the use of an unvarying source of prothrombin (for each group of experiments); *2*) a weak preparation in the incubate corresponding to perhaps 3 to 6 "Iowa" units (see below) per ml; *3*) varying rates and yields according to the activators (and/or inhibitors) being tested; *4*) comparing endpoints, often by simple inspection, for the comparative series *or 5*) comparing with a series of empirical standards (as in any other clotting factor assay). For instance, the last feature mentioned proved very satisfactory in clinical studies on plasma prothrombin, correlating excellently with the Ware-Seegers (19) two-stage and with our specific one-stage (3). Last year, we presented data (4) to illustrate, in principle, how this two-stage test could be modified to quantitate any individual factor involved in thrombin formation. Just as tissue thromboplastin or cephalin (prothromboplastic phosphatide) can be selected for the various specific modifications of the one-stage, so, too, the two-stage may be similarly modified. The fundamental principle is simply that if the system is set up to be deficient in just one factor at a time, it is practicable to use it for the bioassay of that single factor. The very important advantage of our two-stage is that it permits quantitative control of all factors at the same time. This is achieved by independently assaying for each factor in all the reagents (by one-stage or two-stage methods). This kind of control is essentially new and can explain many results of earlier studies, in our own and in many other laboratories, about which we could only guess in the absence of such total factor control. In a "complete" two-stage system, that is, with a fixed (small) amount of prothrombin and optimal (or nearly so) amounts of every one of the other factors needed, thrombin formation is maximal in 2 to 3 minutes, typically. By keeping all accessory factors optimal, except the one tested, the quantitations of additions of the test factor are extremely reliable and specific. Some details of the needed reagents are discussed in Reference 4, and the same conference proceedings include pertinent comments of the late lamented Dr. Hans Jensen, on the technical difficulties in standardizing these test systems. The other type of two-stage was developed by Smith, Warner and Brinkhous (20) and modified by Ware and Seegers (19). This may be termed the "Iowa" method, with its "Iowa (pro)thrombin units." The special feature of this method is to reach a fixed (15 second)[1] endpoint (or almost), varying the dilution of the prothrombin in several steps prior to and during the activation. Total dilution × 1 (or correction factor)[1] constitutes the Iowa unitage. This method obviously does not permit simultaneous quantitative control of all factors in the thrombin-forming system. Ware and Seegers (21) did use it for AcG (V) assays, according to the same principles as our new modifications (4), except for lack of quantitation of the other accessory factors. Seegers and colleagues have published an important and impressive body of work on very many aspects of prothrombin activation and so forth. We would like to point out that, in the many instances [e.g., citrate-activation (22)] in which prothrombin of high purity and very great potency is activated (or altered), the final testing for prothrombin and thrombin unitage does not tell anything about effects which could be due to trace contaminants of even the very best prothrombin preparations. Indeed the excessive amount of prothrombin itself could make it impossible to detect, by independent testing, the presence of possible trace impurities. It is very clear from many workers' experiments, that some, at least, of the accessory factors (which normally accompany prothrombin in plasma) are active in very high dilutions. We have ourselves (23) experienced the complete "spontaneous" activation (on simple standing for several months in solution in the refrigerator) of some of Seegers' prothrombins. We could only attribute this to trace contaminations with all the necessary activators (1). Moreover, Alexander (24) has obtained prothrombin, comparable in purity and potency to Seegers' preparations, in which citrate activation did not occur, until some accessory factor (e.g., proconvertin) was added. Others have similar data, so that there is currently considerable uncertainty as to exactly what factors are essential for thrombin formation. This question of trace contaminants becomes very important in trying to carry our present theme, on the role of enzymes in thrombin formation, to highly purified prothrombin (and so forth) preparations. A further word will be said on this in the Discussion.

METHODS AND MATERIALS

These are fully described, for the most part, in previous publications (1, 3). Test procedures, based upon the principles discussed in the Foreword, include: *1*) whole blood clotting time (CT), in glass (A) or in silicone (B); *2*) plasma recalcification clotting time (PCa); *3*) Ca-tissue thromboplastin-plasma clotting

[1] Only when the endpoint is 15 seconds is the multiplication factor 1, otherwise a correction factor is needed.

time (PT), after Quick, Stanley-Brown and Bancroft (12); *4*) Ca-cephalin-plasma clotting time (PTT), after Langdell and co-workers (13), using substrate plasmas from *a*) normal (N), *b*) hemophilic (VIII- or AHF-), *c*) Christmas disease (IX- or PTC-) or *d*) Hageman-deficient (Hag-) subjects (25); *5*) specific one-stage plasma tests for clotting factors—(VII + X), i.e., "proconvertin" in the wider sense, after Owren (15); VII (SPCA or proconvertin proper) and X (Stuart), separately assayed on appropriate patients' plasma substrates; II (prothrombin); V[AcG or (pro)accelerin]; *6*) specific two-stage tests, on eluates (containing prothrombin and so forth), activated with cephalin (instead of tissue thromboplastin) for the special purposes discussed in the Foreword (4); *7*) other tests, as mentioned in the text. In many experiments, trypsin was tested in combination with (VII + X) (see below) in order to study a "synergistic" phenomenon (26).

Substrates. A routine for human arm-vein blood collection and plasma separation is described in the Foreword; dog blood is handled similarly. Bovine blood is collected in bulk at the slaughterhouse, flowing from the neck section too freely to permit significant tissue contamination. Suitable clinical cases provide factor-deficient plasmas and fractions (1). Artificially prepared substrates (3) include: one-stage prothrombin-(ProT-) substrate; V(AcG)-substrate ("aged" human plasma); VII- and X- (ProC-) substrate [Owren's (15) asbestos-filtered bovine plasma]; plasma eluates obtained with the $BaSO_4$-citrate or $Al(OH)_3$-phosphate (and dialyzed) techniques (1). B. sal., imidazole-buffered NaCl (pH 7.3) was the routine solvent and diluent.

Trypsins. Tryp. A, Worthington's, twice crystallized, salt-free, after Kunitz and Northrop (1936); Tryp. B, a similar commercially available preparation from the Mann Co; Tryp. C, ordinary ("1–300") commercial (N.B. Co.) trypsin. In our test systems, very small concentrations of trypsin suffice and we cannot detect any significant differences between crystalline and "1–300" trypsin, except for the greater potency of the former.

Clotting factors. In advance of the distant goal of biochemical purification of individual clotting factors, we rely upon fractions meticulously tested for all factor activities which might conceivably affect their behavior in the various clotting systems in which they are used. This works out very well in actual practice. (VII + X) was a bovine serum proconvertin, fractionated (in 1955) by our earlier procedure (27), and maintained in frozen-storage at − 20° C. It contained a trace of prothrombin, which the standard (19) two-stage assays showed to be < 5 per cent of that in normal plasma. This was all but negligible in the 1:10 or 1:50 dilutions used for most of the special experiments. Some other *special preparations* will be described in appropriate sections of the text.

RESULTS

Whole blood clotting tests. Table I serves to indicate coagulant activities in a heterogeneous group of agents, the elucidation of which is the project of these studies. Characterization of most of these agents will be deferred until the specific later presentations. The Tryp. A is relevant to the present communication and is described under Methods. The reader's curiosity as to the special *"thrombin"* (T_8) may be satisfied, for the time being, by stating that this was a pool of canine plasma $BaSO_4$-citrate (0.2 M) eluates which had undergone complete spontaneous conversion to thrombin over several years in the deep-freeze (− 20° C); besides thrombic activity, this solution was rich in proteolytic activity. Results similar to those in Table I have been obtained, repeatedly, in glass as well as in silicone. Table II is a similar experiment with varying concentrations of crude trypsin (Tryp. C) tested (A) in glass, (B) in silicone. Clot-retraction and clot lysis were also followed. The former was normal (N) in all tests. Lysis was complete (+) with the 500 μg per ml trypsin in 24 to 48 hours, and incomplete (±) or absent (∞) in the other dilutions. Natural trypsin inhibitors in the blood undoubtedly lessen the enzyme's activity. Clot acceleration was evident even with 5 μg per ml of the crude trypsin and was more marked with the higher enzyme concentrations. The finding of

TABLE I

Effects of various agents on whole blood clotting time, in silicone *

Test	Agent added	Strength	Clotting time
			min
1	B. sal. (control)		24
2	Trypsin A	20 μg	9
3	Papain	1:2500	10.5
4	Bothropase	1:100	7
5	Stypven	1:100,000	3
6	Thrombin (T_8)	0.01 unit	8.5
8	Plasmin B	10 units	7

* Clotting times, in minutes, at 37° C, of 1.8 ml arm-vein blood added to 0.2 ml of agent in buffered (pH 7.3) saline at the strengths stated (per ml of final clotting mixture).

TABLE II

Whole blood clotting time in minutes *

Trypsin (mg/2 ml)	1.0	0.2	0.1	0.02	0.01	0
(A) Glass	4½	7½	7¼	9½	12	13¾
(B) Silicone	4½	7½	9¼	11¾	14	20
Clot-retract.	N	N	N	N	N	N
Clot lysis	1–2 days	±2 days	∞	∞	∞	∞

* Additions of trypsin, 0.2 ml, to 1.8 ml blood at 37° C.

TABLE III

Effects of crystalline trypsin, (VII + X) and a "combination," in routine nonspecific one-stage plasma clotting tests *

Test	Additives	PCa	PT	PTT
1	0 (B. sal.)	189	13.1	86.1
2	Tryp. A, 5 μg	79.4	12.6	50.1
3	(VII + X) 1:50	108.1	12.7	72.7
4	Tryp. A + (VII + X)	32.4	9.4	14.2

* Clotting times in seconds, at 37° C.

TABLE IV

Trypsin, (VII + X), and a "combination," in specific one-stage tests for V(AcG), II(Pro T), and (VII + X), after Owren (15) *

Test	Additives	V(AcG)	II (Pro T)	(VII + X)	Units
1	0 (B. sal.)	87.5	104.5	145.1	0
2	Tryp. A, 2 μg	90.3	108.5	91.8	4
3	(VII + X) 1:50	88.7	73.2	30.3	20
4	Tryp. A + (VII + X)	75.4	78.3	13.2	200

* Clotting times in seconds, at 37° C. Units (last column) as described in text.

almost the same degree of clot-acceleration in silicone as in glass, is suggestive evidence that it does not require SF (see Foreword), which is currently (8, 9, 11) believed to depend upon a PTA-Hageman factor interaction.

Nonspecific one-stage plasma clotting tests. See PCa, PT and PTT under Foreword and under Methods. Table III presents results of these three routine and special tests on a single batch of normal fresh citrated human plasma. Each test used 0.1 ml plasma; 0.1 ml of the combined special additives [containing 5 μg Tryp. A, and 1:50 dilution (VII + X), where indicated]. The "combination," in Tests 4, was preincubated (see below) for 1 minute at 37° C before adding the substrate plasma and the routine activators. The data of Table III show a small, but significant, clot-accelerating action of the 5 μg of trypsin (Tests 2) in all three tests. The very minor effects of the (VII + X), in Test 3, could be due to the trace of prothrombin noted under Methods. The "combination," Test 4, however, evinced a dramatic "synergism," which represents a new and very interesting phenomenon. It is probably significant that the synergized clotting times remain longest in the PCa test, that is, in the absence of added cephalin or tissue thromboplastin. With the combined additives, the cephalin (PTT) test gave values of the order usually encountered with tissue thromboplastin (PT) tests. The PT test, however, could be boosted to super-normal values.

Specific one-stage clotting tests (see Foreword). Table IV shows typical results in testing (3) for V(AcG), II(ProT), and (VII + X), the last by the Owren (15) method. Appropriate deficient plasma substrates (see Methods) were used with the routine Ca-thromboplastin activation, and special 0.1 ml additions of trypsin (Test 2), (VII + X) as above (Tests 3), and the combination (Test 4). The same 1:50 dilution of (VII + X) was used as in the Table III tests, but the trypsin (Tryp. A) was reduced to 2 μg, with a preincubation period (see below) of 3 minutes. There were no significant effects of the trypsin, in Test 2. The 20 unit (VII + X), in Test 3, was inconsequential in the other assays, i.e., nil in V, and merely suggestive, in II, of the trace of contaminating prothrombin. The combination, in Test 4, was also ineffectual in the V(AcG) and II(ProT) tests. In the Owren (VII + X) test, however, it reproduced the same synergistic phenomenon as that previously found in the nonspecific tests (Table III). In the last column are given the percentage assay values, which show a tenfold increase in unitage (Test 4 vs Test 3) when the trypsin reacts with the (VII + X). For these assays, the same preparation of (VII + X) was used as the standard, a 1:10 dilution being designated 100 per cent and a series of dilutions tested to provide data for a curve of reference. The actual test data are reproduced in Table V.

Effects of preincubation of trypsin with (VII +

TABLE V

(VII + X) dilutions tested for use as reference standard in connection with (VII + X) tests of Table IV *

Units.....	200	100	75	50	30	20	10	5	1	0
Clotting time	13.2	14.2	15.6	17.7	21.7	30.3	54.1	88.5	123.2	145.1

* Clotting times in seconds, at 37° C. Units as percentages (100 = 1:10 dilution).

TABLE VI

Preincubation of trypsin with (VII + X), tested by the Owren one-stage (as in Table IV)

Preincub. time (min).....	0.5	1	2	3	5
Clotting time (sec).....	18.7	16.6	15.5	13.2	13.9
Units (from Table V)	44	60	77	200	130

X) received preliminary study, in order to determine suitable concentrations of trypsin and optimal incubation periods. Table VI cites these data for the particular agents used in the experiments of Table IV. There is clearly an optimal incubation period of about 3 minutes and important evidence, in the last test, of instability of the synergistic activity beyond the optimum. The optimal preincubation period, in a series of such studies, seemed to be independent of the (VII + X) dilution, but varied with the trypsin concentration. With 5 μg trypsin (as in Table III) 1 minute was sufficient, and higher enzyme strengths required only a few seconds.

Special one-stage tests, on plasmas specifically deficient in factor X or in factor VII, are included in Table VII. The first four experiments were preliminary data secured with crude trypsin (Tryp. C). Tests 5 through 10, with crystalline trypsin, were performed many months later, with the same preparation (Case L.B.) of VII-deficient plasma (frozen-stored), but with another case (A.G.) with X-deficiency. *Reagent X* (Stuart) was purified (rerun) by continuous flow paper curtain (Spinco) electrophoresis (28), and contained no VII or other detectable clotting factor than X. Its specific potency was tested on plasmas from Case A.G. and from the original R.S. (Stuart), which was used in the first test series. *Reagent VII + X*, 1:50, was the same

TABLE VII

Special one-stage tests on X- and VII- plasmas, adding trypsin, diluted Stuart (X) and (VII + X) preparations *

Test	Additives	X-	Units	VII-	Units
1	0 (B. sal.)	41.3	3	75.8	<1
2	Tryp. C (200 μg)	38.8	4	61.3	<1
3	X	21.9	50	60.1	<1
4	Tryp. C + X	18.8	80	60.0	<1
5	0 (B. sal.)	62.1	0	46.5	1
6	Tryp. A (5 μg)	49.8	<1	36.5	5
7	X	26.7	24	43.5	2
8	Tryp. A + X	22.0	48	32.5	8
9	(VII + X) 1:50	33.5	9	27.7	14
10	Tryp. A + (VII + X)	12.1	>200	16.9	65

* Clotting times in seconds, at 37° C. Units from standard reference curves.

TABLE VIII

PTT tests on Hag-, AHF (VIII)-, and PTC (IX)- plasmas *

Test	Additives	Hag-	AHF (VIII)-	PTC (IX)-
1	0 (B. sal.)	317.6	207.8	800
2	20 μg Tryp. A	38.1	28.2	50
3	2 μg Tryp. A	227.3	82.6	336
4	1:50 (VII + X)	226.9	171.5	518
5	2 μg Tryp. A + (VII + X)	14.9	8.7	9.7
6	*Hag*	47.8		
7	*Hag* + Tryp. A, 2 μg	47.9		

* Effects of trypsin, (VII + X), and a "combination." *Hag* preparation also tested, with and without trypsin, in the Hag- test series. Clotting times in seconds, at 37° C.

as that used in preceding experiments. The results show: *a*) no significant effects of the trypsins alone, in Tests 2 and 6; *b*) good correction of the X-, but not the VII-, tests by the purposely diluted Stuart (X) reagent in Tests 3 and 7; *c*) a definite synergism in Tests 4 and 8, on the X-, but not on the VII-, substrate. In the former, factor VII is present in the substrate and factor X is added; in the latter, the factor VII deficiency remains. When (VII + X), purposely weak, is substituted for the X-reagent, giving the small corrections seen in Test 9, there is a very marked synergism with trypsin, Test 10, in the case of both substrates.

One-stage PTT tests on Hag-, AHF (VIII)-, and PTC (IX)-plasmas (see Foreword). Substrates were obtained from well studied cases. Table VIII shows results of these specific tests upon the addition of: *a*) crystalline trypsin (Tryp. A) in 20 μg (Test 2) or 2 μg (Test 3) amounts; *b*) *(VII + X)* 1:50 (Test 4); or a combination of the last with 2 μg Tryp. A (preincubated for 3 minutes) in Test 5. The results show that the clot acceleration in all three tests by 20 μg per ml trypsin was reduced to slight significance at the level of 2 μg trypsin. The 1:50 (VII + X) also had extremely minor effects. But in the combination, Test 5, there was again a most dramatic synergism with all three substrates. These, obviously, are not specific corrections.

In other Hag- tests: a *Hag* [2] preparation gave good correction, alone (Test 6), and this was unaffected by combining it with 2 μg of crystalline trypsin (Test 7). The *Hag* [2] preparation was obtained by fractionation of recalcified human bank blood (outdated) according to Ratnoff's procedure (25).

[2] See Table VIII, Tests 6 and 7.

TABLE IX

Eluate two-stage tests: effects of trypsin, with various specific factor deficiencies (25° ± 1° C)

Test	Trypsin	Defic.	Incubation period: 0.5 min	Incubation period: Opt. (min)	
			sec	*sec*	*min*
1	—	0	240	23.2	6
	+	0	41.7	23.5	3
2	—	Ca	∞	∞ in 30	
	+	Ca	∞	∞ in 30	
3	—	Ceph.	∞	690 in 30	
	+	Ceph.	1,010	194 in 30	
4	—	V	600	30.6	30
	+	V	610	102.	18
5	—	VII	345	70.5	10
	+	VII	330	76.8	14
6	—	X	210	35.4	7
	+	X	230	37.4	10
7	—	Hag	195	34	7
	+	Hag	87.8	30.3	4
8	—	VIII	480	38.4	9
	+	VIII	88.9	33.2	5
9	—	IX	210	55.7	7
	+	IX	92	49.9	5

Two-stage tests. The principles of these have been reviewed in the Foreword. Technical details are omitted, since these are available in cited references (1, 4, 23). Here we merely omit (−) or add (+) trypsin in the thrombin-forming incubate, which is composed of eluates (containing prothrombin and so forth) and additives. From the standard (5 ml) volume of incubate at room temperature (averaging 26° C), 0.2 ml samples are added at successive intervals to 0.2 ml fibrinogen and the clotting time noted. The 0.5 minute and endpoint (optimal) times are given in Tables IX and X, and inspection of the data readily reveals any significant action of the trypsin.

Various factor deficiencies, with and without added trypsin, were tested by the eluate two-stage, with the results summarized in Table IX. Crude tryp. C, 200 μg per 5 ml incubate, was used throughout in these early (1959) experiments, and the factor deficiencies were *a*) artificial (i.e., omitting the routine additive) in Tests 2 (Ca-), 3 (Ceph-) and 4 (V-); and *b*) clinical cases, in Tests 5 (VII-; *L.B.*),[3] 6 (X-; *A.G.*), 7 (Hag-; *L.C.*), 8 (VIII-) and 9 (IX-). Tissue thromboplastin was used in Test 5.[3] All others were cephalin-activated.

Test 1, with a normal (0 deficient) system, showed a much shorter 0.5 minute test and only one-half the optimal incubation period with trypsin, as compared with the control; the endpoints were the same, however. Thus, trypsin does not make more thrombin, but merely speeds up the activation. In Test 2 (Ca-), no detectable thrombin formed, with or without trypsin, at least in 30 minutes. The action of trypsin requires Ca^{++}. In Test 3 (Ceph-), there was again virtually no thrombin formation, indicating need for the prothromboplastic lipid. In Test 4 (V-), the eluate was heated to 52° C for 30 minutes and the AcG addition omitted. An exceedingly small trace of AcG suffices for some thrombin formation, and an absolute V-deficiency is not easily obtained (29). Nevertheless, the activation here was all but negligible and trypsin, if anything, made it worse (especially the endpoint), perhaps destroying some of the residual traces of AcG. Specifically separate VII- and X-deficiencies were present in Tests 5[3] and 6, respectively. In each case, trypsin failed to improve the inadequate thrombin formation. Trypsin, therefore, needs both of these factors also. In Test 7 (Hag-), 8 (AHF-) and 9 (PTC-), there was a different pattern of results, returning to that found in Test 1 (nondeficient). Thus, when trypsin was added, thrombin forma-

[3] See Table IX.

TABLE X

Two-stage tests on eluates (see text) from Normal, X- (Mr. Stuart), and VII- (L.B.): effects of adding trypsin, (VII + X), or a "combination" *

Test	Additives	Normal: 0.5 min	Normal: Opt.		X-(Stuart): 0.5 min	X-(Stuart): Opt.		VII-(L.B.): 0.5 min	VII-(L.B.): Opt.	
		sec	*sec*	*min*	*sec*	*sec*	*min*	*sec*	*sec*	*min*
1	0	271.5	24.9	10	267.2	27.8	22	345	70.5	10
2	Trypsin	187.4	24.0	7	171.1	31.5	30	330	76.8	14
3	(VII + X)	158.9	25.7	8	129.0	25.8	10	180	26.0	7
4	Tryp. A + (VII + X)	30.3	20.0	2	34.4	22.2	3	102.4	21.1	8

* Clotting times in seconds, after incubation periods of 0.5 minute and at the optimum, in minutes, at 25° C.

tion was distinctly accelerated and improved, despite the specific deficiencies, respectively, in Hag, VIII, or IX. Hence trypsin does not need these three factors for its action in assisting thrombin formation. While we have not yet had the opportunity to study PTA specificially, there are some indications that this also will turn out to be nonessential for the clot-aiding action of trypsin.

Special two-stage tests. Table X gives results on the effects of adding trypsin, (VII + X), or a combination, to activated eluates: *a*) Normal, *b*) X- (Mr. Stuart), *c*) VII- (L.B.). In each case, 0.1 ml of 1:5 dilution of the corresponding fresh supernate, after the $BaSO_4$ adsorption of the plasma, was added to provide AcG, AHF, PTA, Hageman, and the AcG(V) was further boosted by 0.1 ml of a 1:10 dilution of $BaCO_3$-adsorbed beef serum. One-tenth ml of eluate was used in all tests. The (VII + X) was 0.1 ml of a 1:10 dilution in each case, but the 0.1 ml trypsin differed, viz., *a*) 5 μg Tryp. B; *b*) 20 μg Tryp. A, *c*) 200 μg Tryp. C. One-tenth ml tissue thromboplastin in *c*),[4] or ceph. (human brain cephalin, 0.15 per cent) in *a*) and *b*), and 0.5 ml Ca (0.15 M $CaCl_2$) were the routine activators, and all incubates were made up to 5 ml with b. sal.

The major findings were: *1*) trypsin, alone, in the Normal significantly improved the *rate* of thrombin formation, as shown by the initially (0.5 minute) shorter clotting-time and by the reduction in the optimal incubation period needed, whereas, the enzyme failed significantly to improve the activations in the X- and VII- incubates. *2*) Trypsin, with (VII + X), markedly improved the activations in Test 4, in which the special additives were preincubated for 1 minute before adding the other components. Minor technical imperfections are apparent in these tests, but do not detract from the significant major conclusion, confirmatory of the results with the one-stage tests, that trypsin acts synergistically with *both* VII and X.

Lack of influence of trypsin on the fate of factor VII activity in shed Stuart (X-) blood. The Quick (PT) test (12), Owren's (VII + X) test (15), or specific one-stage factor VII assays (30) show a remarkable phenomenon concerning the changes of factor VII activity in shed *normal* blood. Activity values up to 200 to 300 per cent of the original plasma level are frequently encountered after withdrawing the blood sample and allowing it to clot (in glass) at 37° C. Factor X behaves quite differently, showing only minor fluctuations essentially indicating no change from the original plasma level (30). In VII- or X- cases, the respective missing factor would not be expected to arise in the serum *de novo*, and data are available to confirm this. There is also no reason to expect the fate of X to be different in VII-deficient blood, and this, too, can be substantiated. It is of interest to follow the fate of VII activity in X-deficient blood. A rare opportunity to do this occurred recently during a visit of the original Mr. Stuart (X-). Specific one-stage factor VII assays were performed, thanks to availability of plasma substrate from Case L.B. (VII-). The Owren test (for VII + X) was also run, but gave very low values throughout, merely indicating that no change occurred which might counteract the deficiency of factor X, which is also needed for this test. Four tubes of blood were incubated with

[4] See Table X.

TABLE XI

Assays for VII, and (VII + X), in incubated Stuart's (X-) blood, with (+) or without (−) trypsin †*

Test	Tryp.	0	5 min	10 min	20 min	1 hr	4 hr	24 hr	Control
VII	−	18.0 [100]	18.4 [94]	18.0 [100]	17.6 [107]	17.0 [116]	16.8 [122]	19.0 [82]	52.0 [0]
VII	+		19.3 [80]	19.1 [81]	19.6 [76]	17.7 [105]			52.0 [0]
(VII + X)	−	61.2 [4]	66.2 [3]	59.8 [4]	54.9 [5]	64.3 [3]	68.6 [2]	51.7 [9]	154.6 [0]
(VII + X)	+		67.9 [2]	58.3 [4]	63.7 [3]	63.1 [3]			154.6 [0]

* Clotting time (37° C): with 20 μg/ml trypsin, 16 minutes; without trypsin, 19 minutes.
† Clotting times in seconds; percentage units in brackets; temperature 37° C.

1/10 volume crystalline trypsin, tryp. A, to 20 μg per ml, the others receiving an equivalent volume of b. sal. Unitages were computed from standard curves with normal human plasma dilutions. The data are given in Table XI. The results show only very minor changes in VII-activity over 24 hours, in the absence of trypsin. This is quite unlike the normal post-clotting rise in VII activity (30). With trypsin followed over 1 hour, the results were not significantly influenced.

This single experiment must be regarded as preliminary, and it is hoped that we, and others, will find similar opportunities for its future repetition and further analysis. Such has perhaps appeared recently in a report by Hougie (31). One of his cases (A.G.) showed results similar to the above, whereas another (identified as "mild," with perhaps some 10 per cent of the normal X) behaved normally.

DISCUSSION

Using microgram amounts of trypsin largely avoids known (1) destructive actions of the enzyme on clotting proteins and brings out some remarkable clot-accelerating effects in a variety of test systems. By the failure of these to occur in factor-deficient systems, it is clear that the action of trypsin requires Ca-ions, a special pro-thromboplastic (4) lipid, such as cephalin, together with factors V, VII, and X, but *not* factors VIII, IX, (PTA), or Hageman. While this is still a complex thrombin-forming system, involving at least seven components, when prothrombin and trypsin are included, it is, nevertheless, somewhat simpler than the natural plasma system, which requires at least four more factors, namely, Hageman, PTA, PTC(IX), and AHF(VIII). We have previously presented an experimental analysis (4) of the mode of action of Russell's viper venom (Stypven), which differs from trypsin in not requiring factor VII. Later papers of the present research will deal with other enzymes and so forth and add further data to strengthen the current body of facts which point to a diversity of possible thromboplastin- and thrombin-forming systems.

Incidentally, plasmin and other thrombolytic agents do not act in these ways, but merely contribute a Hageman-like effect to explain their ability to accelerate blood-clotting (10). Plasmin is *not* a "thromboplastic enzyme."

It will be a long time before the complex multicomponent reactions of blood-clotting permit a genuinely biochemical interpretation. In the meantime, it seems helpful to regard trypsin (and certain other enzymes) as accessory factors in their own right. They work with particular groups of the natural cofactors, and diverse combinations of accessory factors (according to the enzyme) achieve the same end result, namely, thrombin formation. It is premature to speculate what the intermediary pathways may be.

In 1916, Dale and Walpole (32) reported that trypsin "liberated an active thrombokinase" in fowl plasma. Our past work (1) emphasizes key roles for calcium ions and a special lipid (or lipids) which we propose to call "prothromboplastic phosphatide" (4). The term "thromboplastic enzyme" (7), based on experiments with trypsin, indicates catalysis of the "thromboplastin" [if such there be (4)] and thrombin-forming reactions. The new discovery (26) of the remarkable synergistic reaction between trypsin and *both* factors VII and X, may very well be of the most fundamental significance. May it not, for instance, suggest that very small amounts of these accessory factors suffice in the presence of trypsin? Does the effectiveness of trypsin in activating highly purified prothrombins depend upon trace impurities of these cofactors which, perhaps, cannot be detected in any other way? Our previous work with at least one purified prothrombin did not reveal any thrombin-forming activity of trypsin alone or with calcium, but only when (weak) thromboplastin was added (23). Another of our Seegers-type prothrombins, at that time (1947), showed complete activation spontaneously in several months at ice-box temperatures. We could only suggest the presence in this of significant trace contaminants of *all* essential activators (1). In our "complete," or single-factor deficient, present two-stage systems, micrograms of trypsin reveal their effectiveness (or lack of it) in a very few minutes. We make no claims whatever for other than relative factor inadequacies in the current reagents. Hence results of using stronger enzymes and hours of incubation (24) are regarded as another story, in which we would personally raise cogent questions as to possible trace contaminants. Additional complications with larger

amounts of enzyme undoubtedly include destructions of certain clotting factors, perhaps even of thrombin itself (24). The lower and upper limits of effectiveness of each individual clotting factor need to be worked out for each variant of the clotting test system. The permutations and combinations are almost endless, and offer a fruitful field for further enquiry and possible practical applications.

We have tried, without significant results, to combine trypsin with other accessory factors, which are at present available to us only in crude forms. For instance, preliminary two-stage tests employing trypsin with normal $BaSO_4$-adsorbed plasma to restore AHF, or with serum to restore PTC, at first suggested some potentiation, compared with trypsin alone. This led to an error on page 160 of our monograph (1) in which VIII and IX were included among the cofactors needed by trypsin. Rechecking the data revealed that these findings were caused by traces of prothrombin in the added plasma or serum preparations. Factors VIII and IX are *not* needed and correction of the error is herewith requested.

The argument that trypsin can help reveal traces of factors VII and X can be reversed. Is it not possible that the normal increase in VII activity in shed blood could be due to the working of a natural "tryptase" (i.e., trypsin-like, in a broad sense) enzyme in the blood? Our single experiment, on the fate of VII activity in X- blood, needs confirmation and an attempt should be made to restore the normal post-clotting rise in VII activity, simply by adding factor X. On all these matters our ideas are very tentative. We do feel, however, that present indications encourage further exploration of a "thromboplastic enzyme" theory of blood clotting (33).

SUMMARY

Trypsin accelerates blood clotting in a variety of test systems. This requires Ca-ions, cephalin (or equivalent prothromboplastic lipid), together with accessory factors V, VII, and X, but not VIII, IX, (PTA), or Hageman. In combination with both VII and X, trypsin acts with a powerful *synergism*. The further exploration of what lies behind these experimental facts could lead to the establishment of a "thromboplastic enzyme" theory of blood clotting.

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A STUDY OF EIGHT HEATED PLASMA PROTEIN PREPARATIONS

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Human plasma has not gained general acceptance as a therapeutic agent because of the risk of hepatitis following its use. A number of physical and chemical treatments have been tried to minimize this risk but the incidence remains about 0.5 per cent for single donor plasma and upwards of 20 per cent for some lots of pooled plasma (1, 2). Ultraviolet irradiation, which at one time seemed quite promising, proved incapable of inactivating the hepatitis virus at radiation doses which allowed preservation of the integrity of the plasma proteins. Another physical agent, heat, has demonstrated the ability to reduce markedly, if not completely eliminate, the hepatitis risk in human albumin (3). In this situation an albumin solution containing a stabilizer is heated for 10 hours at 60° C. This treatment does not modify the protein to an extent which makes it unsuitable for infusion. After more than a decade of wide use, a substantiated case of hepatitis caused by injection of heated human albumin has yet to be reported.

Just as the usefulness and safety of albumin are well recognized, so also are the problems of its high cost, complexity of production, and relatively small yield as compared to plasma. Because of these factors a number of individual investigators and industrial concerns set out to develop a plasma expander which would be as useful therapeutically as albumin, but which would be less expensive and have no greater risk of transmitting hepatitis. Added impetus was given to this program by the need of the Office of Defense Mobilization and the Department of Defense for a safe plasma expander to be manufactured from their outdating stockpile of plasma.

On the basis of the work of Gellis and associates (3) and of Murray (1) on the heating of albumin, most of the investigators involved decided that a major effort should be devoted to developing a plasma derivative which could be heated without adversely affecting the constituent proteins. Murray and co-workers had shown previously (4) that the heating of whole plasma for 4 hours at 60° C produced gross changes in the proteins and did not eliminate hepatitis infectivity. It was hoped, however, that the evidence for heat inactivation of virus in albumin could be extrapolated to plasma. The main problem was to develop a procedure which would stabilize the proteins, allowing the plasma derivative to be heated for at least 10 hours at 60° C without significant alteration.

The problem was approached in two somewhat different ways. Some of the workers hoped to solve the dilemma by the use of whole plasma to which had been added a chemical stabilizer in order to minimize protein denaturation. Other workers used modifications of the recognized plasma fractionation procedures, intending thereby to eliminate those fractions of plasma which seemed most susceptible to heat denaturation. They hoped that the partially fractionated final product would turn out to be as heat stable and safe as albumin, but less expensive than that preparation.

With the cooperation of the National Research Council, this laboratory had the opportunity of doing simultaneous analyses on these various plasma preparations in an attempt to detect and perhaps evaluate some of the physical and chemical changes which were taking place as a result of the treatments. This report will present comparative data on eight such preparations. When unheated controls were available, they were similarly studied. All materials were also compared with normal unheated citrated plasma.

MATERIALS AND METHODS

As mentioned previously, the preparations were of two general types: heated whole plasma, and heated, partially fractionated plasma protein solutions. Three of the preparations, designated [1], [2] and [3], were in the first group and five, designated [A], [B], [C], [D] and [E], were in the second. The methods of production will be described briefly and are summarized in Table I.

Preparation [1] was made by heating for 10 hours at 60° C plasma which had been irradiated with ultraviolet light according to the requirements of the National Institutes of Health (5) and in which standard acid-citrate-dextrose (6) had been used as an anticoagulant. In preparing [2], serum was filtered, heated at 55° C for 10 hours, refiltered, and then lyophilized. Preparation [3] consisted of plasma in 4 per cent citrate as an anticoagulant to which had been added 5 per cent invert sugar. This solution was irradiated with ultraviolet light, filtered, and then heated at 60° C for 10 hours. It is noteworthy that in two of these three preparations a sugar was used in an attempt to achieve some stabilization of the proteins. In the other, no stabilizer was used, all precipitated protein being removed after heating.

[A] was prepared by the method of Hink, Hidalgo, Seeberg and Johnson (7). This consisted essentially of fractionating plasma by method 6 of Cohn and co-workers (8) with precipitation of fractions I, II + III, and IV-1. The supernatant IV-1 was precipitated with cold ethanol and the precipitate dried. The precipitate was dissolved in a solution of water, 0.112 M sodium chloride, 0.004 M sodium acetyltryptophanate, and 0.004 M sodium caprylate (9), and heated for 2 hours at 60° C, filtered into final containers and then heated at 60° C for 10 hours.

Preparation [B] was made by the method of Mulford, Mealey and Welton (10), in which, following a method 6 fractionation through the precipitation of fraction II + III, the supernatant II + III was filtered, dried, reconstituted to 5 per cent in a solution containing 0.008 M sodium acetyltryptophanate and heated at 60° C for 10 hours. Preparation [C] represents a modification of this same method in which fractions IV and V are alcohol precipitated from supernatant II + III with 40 per cent ethanol at pH 4.8, dried, redissolved in a solution containing 0.008 M sodium acetyltryptophanate and heated at 60° C for 10 hours.

For the preparation of [D], plasma was first precipitated with 45 per cent saturated ammonium sulfate. The supernatant was treated with a mixture of fatty acids, filtered, and then precipitated with 70 per cent saturated ammonium sulfate. The precipitate was redissolved in saline, 0.04 M sodium caprylate added, and the solution heated at 60° C for 10 hours (11). The method of Surgenor and co-workers (12) was used for preparing [E]. This utilized a zinc precipitation technique to remove fibrinogen, γ-globulin, and some β-lipoproteins. Zinc was removed from the supernatant by an ion exchange resin, following which the solution was heated at 60° C for 10 hours in the presence of sodium caprylate. This material is frequently called "stable plasma protein solution."

Our purpose in carrying out this study was to provide a preliminary evaluation of these products that would help in determining which ones, if any, would merit more detailed, intensive investigation. With this in mind, the following procedures were included in this preliminary screening: gross examination, testing for sterility and pyrogen according to the requirements of the National Institutes of Health (13), protein concentration by micro-Kjeldahl, nonprotein nitrogen, sodium, potassium, thermal stability, electrophoresis, and ultracentrifugation.

Thermal stability was evaluated by the method described in Reference 13, using an Aminco light-scattering microphotometer. Incubation conditions of 50° C for 12 days, 57° C for 50 hours, and −20° C for 24 hours for separate samples were utilized. Electrophoresis was performed by the moving boundary method in an Aminco-Stern apparatus, using barbital buffer at pH 8.6 and 0.1 ionic strength. Mobilities were determined in the usual manner, and relative proportions by the method of completion of the curves and planimetry. Ultracentrifugation was performed on a Spinco model E ultracentrifuge using the sedimentation velocity technique on the protein solution dialyzed against phosphate buffered saline at pH 7.2, 0.154 ionic strength. Sedimentation coefficients and relative proportions were both computed. Sodium and potassium determinations were made by flame photometry on a Baird flame photometer.

RESULTS AND DISCUSSION

The requirements of a plasma expander to be held in a stockpile would seem to be threefold: *1*) the material must be safe for infusion in large amounts and at frequent intervals; *2*) it must be able to withstand storage and shipment under a considerable range of temperature without undergoing significant changes in the constituents; and *3*) it must provide the therapeutic effect desired, namely, blood volume expansion. The investigation described herein is an attempt to provide a preliminary indication of the ability of these preparations to fulfill the first two demands. The third is best evaluated by actual clinical trial and thus merits investigation only after the safety and stability of the material are proved.

The sterility tests failed to demonstrate bacterial contamination in any of the preparations. In none of the preparations was the pyrogen reaction greater than that usually considered acceptable.

Simple inspection revealed interesting information on several preparations. A tabulation of the gross appearance of the various materials is shown in Table I. In preparation [3] a progressively increasing reddish-brown color was noted. A much less marked darkening was also noted in preparation [1].

The contrast between preparations [B] and [C] is also pertinent. When received, [B] was found to have a white sediment at the bottom of the container. No such sediment was present in preparation [C]. Information received from the manu-

TABLE I

Methods of preparation and gross appearance

Whole plasma products	Fractionated plasma products
[1] Acid-citrate-dextrose plasma, ultraviolet treatment, heat 10 hours at 60° C Heated—yellow, slightly opalescent solution Unheated—yellow, slightly opalescent solution	[A] Supernatant IV-1, precipitate with ethanol, redissolve precipitate in solution containing stabilizer, heat 2 hours at 60° C, filter, heat 10 hours at 60° C Heated—pale yellow, clear solution Unheated—pale yellow, clear solution
[2] Serum, heat 10 hours at 55° C, filter Heated—yellow, clear solution Unheated—none available	[B] Supernatant II + III, add stabilizer, heat 10 hours at 60° C Heated—yellow, slightly opalescent solution with white sediment Unheated—none available
[3] 4% Citrate plasma, 5% invert sugar, ultraviolet treatment, heat 10 hours at 60° C Heated—yellow, clear solution, becoming progressively redder during storage Unheated—none available	[C] Fractions IV + V, add stabilizer, heat 10 hours at 60° C Heated—yellow, clear solution Unheated—none available
	[D] Supernatant of 45% $(NH_4)_2SO_4$ saturation treated with mixed fatty acids, filtered, precipitate with 70% saturated $(NH_4)_2SO_4$, redissolve and add stabilizer, heat 10 hours at 60° C Heated—amber, clear solution Unheated plasma—slightly cloudy, yellow brown solution
	[E] Zinc precipitation, removal of zinc from supernatant, add stabilizer, heat 10 hours at 60° C Heated—clear, yellow solution which became progressively more cloudy during storage at 5° C Unheated—none available

facturer indicated that both preparations were clear and without sediment before shipping. To check this, another sample of [B], known to be without sediment just before shipment, was sent to us and found to have a significant amount of white sediment on receipt. It was evident that rather slight changes in the method of preparation could be responsible for a significant difference in the ability of a preparation to undergo shipment without alteration. The difference between [B] and [C] is further emphasized by examination of the ultracentrifugal patterns of the two preparations (Table V and Figure 4). Rapidly sedimenting components are present in both [B] and [C]. The rapid component in [C], however, has a sedimentation coefficient significantly lower than the comparable one in [B], and represents less of the total protein present. Subsequent pictures in each of the runs seem to indicate that the fast moving component consists of a great number of smaller components, each with a slightly different sedimentation coefficient.

The protein, nonprotein nitrogen, sodium, and potassium values are listed in Table II. All of the preparations, except [D], had protein concentrations approximately equal to, or exceeding that of plasma. Preparation [D], which had a concentration slightly lower than the others, could easily be prepared in a manner that would raise its concentration to a level comparable to that of the others.

TABLE II

Results of protein, nonprotein nitrogen, pH, Na^+ and K^+ determinations

Product	Protein	NPN	pH	Na+	K+
	g/100 ml	*mg/100 ml*		*mEq/L*	*mEq/L*
[1] Heated	5.11	20	7.82	192	0.2
[1] Unheated	5.02	26	8.08	192	0.3
[2] Heated	6.92	17	8.05	135	5.1
[2] Unheated	7.00	18	7.96	134	7.0
[3] Heated	5.28	17	7.61	186	4.0
[A] Heated	4.72	20	7.02	134	1.4
[A] Unheated	4.87	20	6.93	134	1.4
[B] Heated	4.89	41	6.64	235+	18.9
[C] Heated	4.57	21	7.90	235+	Trace
[D] Heated	3.60	12	6.41	143	Trace
[D] Unheated plasma	6.87	42	7.44	130	6.7
[E] Heated	5.25	52	7.78	234	Trace

TABLE III

Thermal stability estimated nephelometrically *

	50° C			57° C			−20° C		
	Initial	After 12 days	Change	Initial	After 50 hrs	Change	Initial	After 24 hrs	Change
[1] Heated	192	314	122	191	341	150	272	Too turbid to read	?
[1] Unheated	144	276	132	136	285	149	116	169	53
[2] Heated	137	384	247	115	Soft gel	?	197	Too turbid to read	?
[2] Unheated	85	345	260	82	Soft gel	?			
[3] Heated	84	100	16	77	124	47	81	163	82
[A] Heated	22	25	3	20	21	1	23	53	20
[B] Heated	130	108	−22	141	103	−38	172	172	0
[C] Heated	106	100	−6	92	107	15	125	179	54
[D] Heated	6	10	4	6	8	2	9	13	4
[E] Heated	145	Too turbid to read	?	134	Too turbid to read	?	Cloudy after 2 wks in refrigerator at 3° C (too turbid to read)	Striking increase in turbidity	?

* Units are arbitrary "nephelometric units" (13).

Preparations [B] and [E] had nonprotein nitrogen values which were appreciably higher than those found in any of the other materials. There seems to be some correlation in samples [B], [C], and [E] between lack of thermal stability, or lack of stability on shipment, and higher nonprotein nitrogen values. It is of interest that although [B] and [C] had the same amounts of sodium acetyl-

TABLE IV

Moving boundary electrophoresis

Material	Component	Mobility*	Relative proportion	Material	Component	Mobility*	Relative proportion
			%				%
[1] Heated	a	−6.7	49.3	[A] Heated	a	−6.8	86.6
	b	−4.6	46.5		b	−5.2	11.3
	c	−3.3	0.9		c	−3.5	2.0
	d	−1.6	3.3	[A] Unheated	a	−6.5	85.2
[1] Unheated	a	−6.6	62.5		b	−4.4	5.3
	b	−5.6	8.8		c	−3.4	9.2
	c	−4.3	12.0				
	d	−3.2	6.3	[B] Heated	a	−6.4	85.2
	e	−1.3	10.4		b	−4.8	13.6
					c	−3.2	1.2
[2] Heated	a	−6.4	50.0				
	b	−5.4	2.9	[C] Heated	a	−6.8	81.4
	c	−4.0	26.6		b	−5.2	17.2
	d	−3.2	10.4		c	−3.2	1.3
	e	−1.2	10.2				
[2] Unheated	a	−6.2	58.1	[D] Heated	a	−6.1	88.5
	b	−5.3	4.7		b	−4.8	6.8
	c	−3.9	12.0		c	−3.0	4.7
	d	−2.8	13.0	[D] Unheated	a	−5.7	60.7
	e	−1.3	12.3	plasma	b	−4.1	9.0
					c	−3.4	8.7
[3] Heated	a	−6.4	49.3		d	−2.4	8.4
	b	−4.6	47.9		e	−1.7	3.1
	c	−1.7	2.8		f	−0.6	10.1
				[E] Heated	a	−6.3	79.0
					b	−4.5	18.2
					c	−3.0	2.8

* Mobility is shown in $cm^2/volt/sec \times 10^{-5}$.

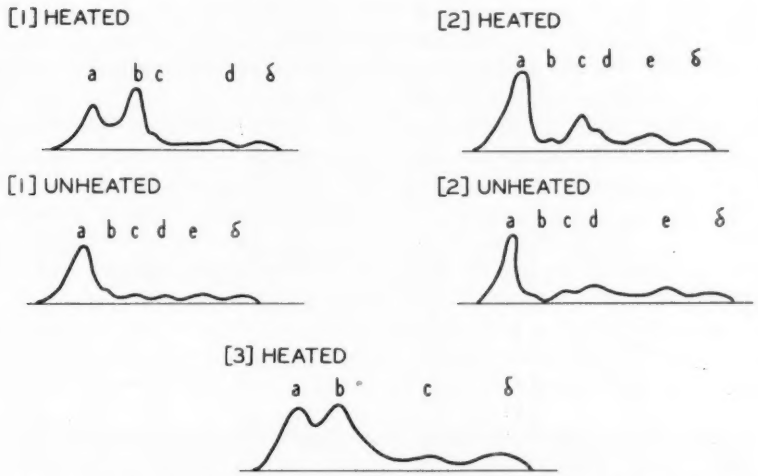

FIG. 1. ELECTROPHORETIC PATTERNS OF WHOLE PLASMA SAMPLES. Direction of migration is from right to left.

tryptophanate added, preparation [C] had a nonprotein nitrogen value only half that of [B]. Preparation [E] had no added nitrogen-containing stabilizer. The significance, if any, of this finding is not clear, but may be an indication of the breaking off of small peptide groups as a result of heating and/or storage of plasma proteins under certain conditions.

The high sodium concentrations in preparations [1], [3], [B] and [E], and the high potassium concentration in [B] give some cause for concern because these values are above both physiological levels and those found in normal citrated plasma. On this basis one would be hesitant about administering them in conditions characterized by sodium retention or renal insufficiency.

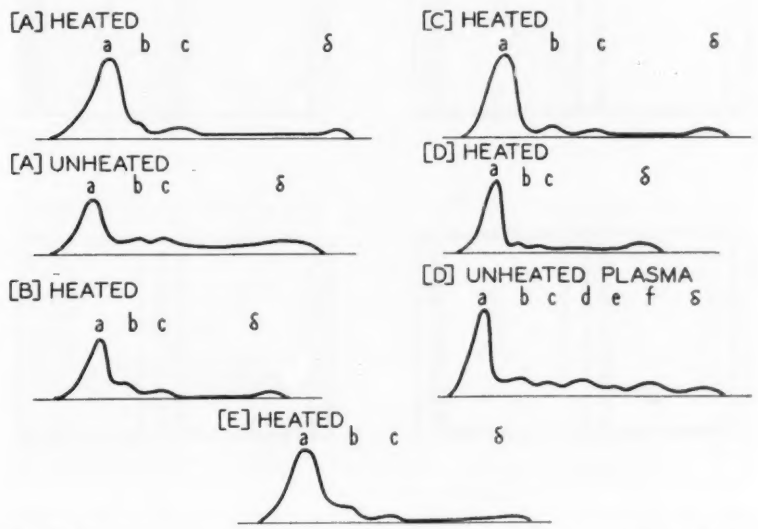

FIG. 2. ELECTROPHORETIC PATTERNS OF FRACTIONATED SAMPLES. Direction of migration is from right to left.

TABLE V

Ultracentrifugal analyses

Material	Component	$s°_{20,w}$	Relative proportion	Material	Component	$s°_{20,w}$	Relative proportion
			%				%
[1] Heated	I	21.0	12	[A] Heated	I	14.0	4
	II	6.6	6		II	3.9	96
	III	4.4	82				
				[A] Unheated	I	3.9	100
[1] Unheated	I	17.5	7				
	II	6.5	15	[B] Heated	I	21.0	8
	III	4.3	78		II	4.0	92
[2] Heated	I	17.6	3	[C] Heated	I	17.0	6
	II	6.8	19		II	3.9	94
	III	4.1	78				
				[D] Heated	I	24.0	2
[3] Heated	I	23.0	13		II	3.8	98
	II	6.7	11				
	III	4.3	76	[D] Unheated plasma	I	16.5	4
					II	6.2	19
					III	4.0	77
				[E] Heated	I	20.0	6
					II	4.0	94

Table III indicates the relative changes in turbidity after exposure to temperatures of 50° C for 12 days, 57° C for 50 hours and − 20° C for 24 hours. All of the heated whole plasma preparations demonstrated generally poor thermal stability at the various temperatures. Also noteworthy is the poor stability in the cold of preparation [E], a situation which could be inferred from the gross observation. Of the fractionated materials, this preparation seemed the least stable

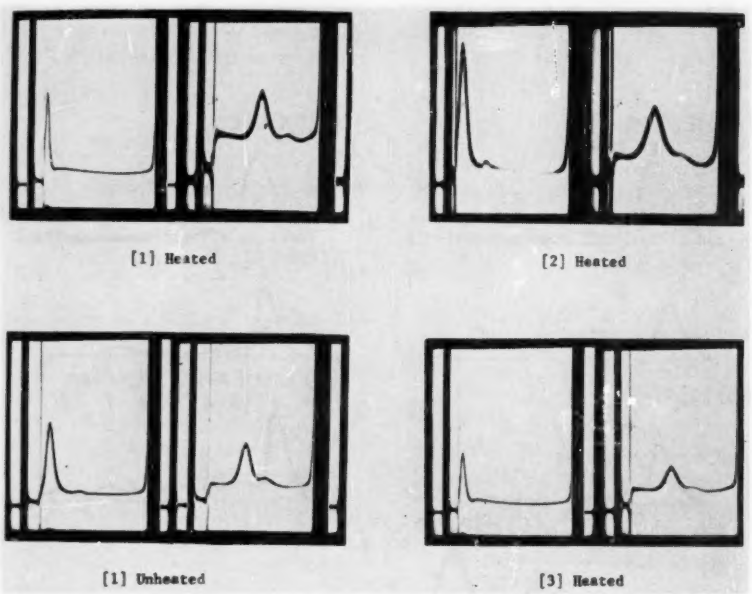

FIG. 3. ULTRACENTRIFUGAL PATTERNS OF WHOLE PLASMA SAMPLES. Times of pictures after attainment of full speed are as follows: [1] heated, 231 and 5,031 seconds; [1] unheated, 853 and 3,973 seconds; [2] heated (at 44,770 rpm), 1,326 and 9,006 seconds; [3] heated, 272 and 4,112 seconds.

at all temperatures. The decrease in the nephelometric reading of [B] after heating is possibly due to enlarging of small aggregates with subsequent sedimentation.

The low temperature instability of the whole plasma preparations is consistent with, and probably a result of lipoprotein denaturation at low temperatures as described by Oncley, Gurd and Melin (14). The low temperature instability noted in [E] is not as readily understandable, but may also be a reflection of the presence of significant amounts of lipoprotein.

The results of the electrophoretic analyses are given in Table IV, the patterns in Figures 1 and 2. These demonstrate marked changes in the heated whole plasmas containing sugar, i.e., [1] and [3]. These changes consist primarily of decreases in proteins moving with mobilities of albumin and β- and γ-globulins, and marked increases in proteins having mobilities comparable to those of α_2-globulins. This is consistent with the findings of Miyamoto, Sakagishi and Sano (15), Ardry (16), and Bozzetti and Prina (17). On electrophoretic examination, preparation [2] seemed relatively less changed than either of the other similar preparations, although there was an increase in the component with the mobility of α_2-globulin. The fractionated preparation on which an unheated

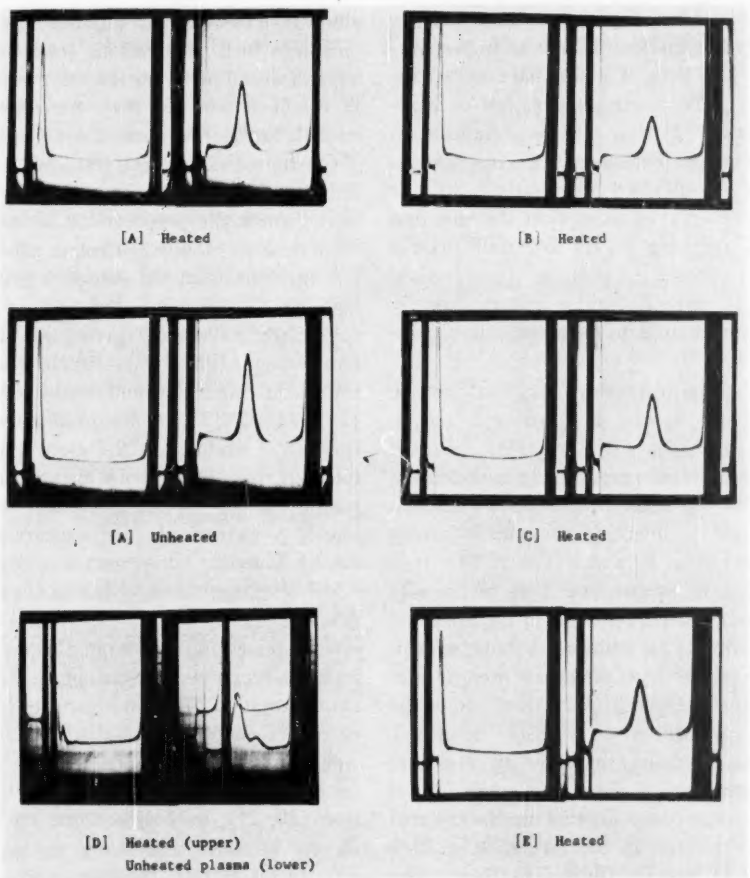

FIG. 4. ULTRACENTRIFUGAL PATTERNS OF FRACTIONATED SAMPLES. Times of pictures after attainment of full speed are as follows: [A] heated, 434 and 4,561 seconds; [A] unheated, 366 and 6,121 seconds; [B] heated, 233 and 5,993 seconds; [C] heated, 169 and 6,167 seconds; [D] heated, 221 and 3,912 seconds; [D] unheated plasma, 221 and 3,912 seconds; [E] heated, 201 and 5,961 seconds.

control was available, [A], demonstrated changes of a much less striking nature in its electrophoretic pattern.

These findings on electrophoresis together with the color changes previously noted may be indicative, among other things, of a type of protein degradation characterized by an initial reaction between the protein and reducing sugars. This type of "browning reaction" occurring in systems containing amine groups and reducing sugars is treated exhaustively by Hodge (18). This reaction may be of great significance in influencing the stability of protein solutions. Suggestive evidence is indicated in the electrophoretic patterns of Figure 1, in which the changes in preparation [2], a material with no added reducing sugar, are significantly less marked than those in preparations [1] and [3], both of which have added reducing sugars. The lower temperature of heating of preparation [2] may also be significant in lessening the changes detected by electrophoresis. A number of unpublished observations in our laboratory, however, tend to support the idea that the addition of reducing sugars to certain protein systems may potentiate protein denaturation. This problem is currently under investigation by our group in an attempt to determine its significance.

The results of the ultracentrifugal analyses are indicated in Table V and in Figures 3 and 4. Preparations [1], [3], [B] and [E] revealed measurable amounts of very rapidly sedimenting material. All of the other preparations exhibited a relatively rapid sedimenting component having an $s^{\circ}_{20,w}$ greater than 10 and less than 18. It is noteworthy that in preparation [A] no rapidly sedimenting component is present in the unheated control. It would be unusual for significant amounts of relatively high molecular weight substance to remain in a preparation after any of the fractionation procedures previously described. One is probably justified, therefore, in assuming that the appearance of significant amounts of rapidly sedimenting component in the fractionated preparations is a result of the formation of high molecular weight species during heating. The presence of relatively large amounts of rapidly sedimenting component in the whole plasma preparations may similarly be attributed to heating or storage, since these components are not usually seen in more than trace amounts in either serum or plasma which has not been concentrated prior to centrifugation.

CONCLUSIONS

The purpose of this study was to attempt to obtain a preliminary indication of the possible potential clinical usefulness of the various heated plasma preparations. It was hoped that on the basis of a few selected physical and chemical tests, some assumptions could be made regarding the safety of the products. Certainly, the final judgment regarding safety can depend only on appropriate intensive immunological, toxicological, and clinical studies. However, an attempt was made to determine which preparations seemed most promising and would merit the intensive studies necessary to establish whether they were safe and potent enough for routine clinical use.

On the basis of these tests, we felt that a certain number of conclusions could be drawn which would allow the preparations to be graded as to their relative promise. Certain products could be put into the doubtful category because of their inability to withstand shipment and/or temperature extremes without giving indications of protein change. These would include at least preparations [B] and [E], and most likely preparations [1], [2] and [3]. Some products were in doubt because of findings in the electrophoretic and/or ultracentrifugal analyses which seemed to indicate major changes in the proteins. We were primarily concerned with the appearance of the rapidly sedimenting component which was present in relatively large amounts in certain of these products. Because of the experience with dextran, which seemed to indicate an adverse effect of very high molecular weight colloidal substances on the clotting system (19), and because of the possibility of reticuloendothelial blockade by high molecular weight abnormal proteins with an adverse effect on the reaction of this system to the shock situation (20, 21), we believed caution was necessary in any *in vivo* preparations testing of [1], [2], [3], [B] and [E], the same preparations put in doubt by the stability studies.

Three of the preparations, [A], [C] and [D], all fractionated materials, seemed the most prom-

ising on the basis of these preliminary physical and chemical determinations. These three preparations demonstrated relatively good stability characteristics, showed less marked changes on electrophoresis and ultracentrifugation than the others, and contained a high proportion of albumin (greater than 80 per cent) which brought them close in character to a material which has already proved clinically safe and effective, normal serum albumin (human).

The clinical significance of the changes noted in any of the preparations is unknown at this time.

SUMMARY

Eight heated plasma preparations were compared by a number of physical and chemical techniques. These tests demonstrated better stability characteristics and resistance to molecular change in those preparations which had undergone a partial fractionation procedure prior to heating. Three preparations produced by different fractionation methods were deemed to be the most promising as possible plasma expanders for human use and meriting further study both *in vivo* and *in vitro*. These were, respectively, a precipitate from supernate IV-1 of Cohn's fractionation method 6, fractions IV + V from Cohn's method 6, and a preparation from the supernate of plasma 45 per cent saturated with ammonium sulfate.

ACKNOWLEDGMENTS

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EFFECT OF CHRONIC HYPOTHALAMIC STIMULATION UPON CHOLESTEROL-INDUCED ATHEROSCLEROSIS IN THE RABBIT *

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The possible role of the central nervous system (CNS) in the pathogenesis of experimental atherosclerosis has received scant investigative scrutiny. Indeed, it can even be stated that the majority of investigators in the field of experimental atherosclerosis are frankly skeptical of any possible etiological relationship between dysfunction of any central neural mechanisms and peripheral atherosclerosis. Certainly in most of the recent American reviews concerned with atherosclerosis the subject of nervous factors in the etiology of atherosclerosis has either been minimized or totally neglected.

Despite this seeming exclusion of the CNS from laboratory studies of atherosclerosis by many investigators, certain experimental and clinical data make this neglect appear rather unfortunate. Myasnikov (1, 2) has demonstrated that when drugs that depress certain CNS mechanisms (e.g., barbiturates) were chronically given to cholesterol-fed rabbits, these rabbits exhibited a significant decrease in hypercholesterolemia and aortic atherosclerosis. On the other hand, phenamine, a drug which excites the CNS, produced the opposite effects, increasing the hypercholesterolemia and atherosclerosis (3). Finally, it has been reported that rats fed an atherogenic diet and exposed to a particular form of stress exhibited greater degrees of hypercholesterolemia and coronary atherosclerosis than their controls fed the same diet (4).

Several clinical studies also strongly suggest that changes in psychological behavior influence not only the level of blood cholesterol (5–8) but also the incidence of clinically manifested coronary artery disease (9).

If these experimental and clinical reports are valid, they force us, despite their paucity, to include the CNS in the ambit of investigation concerning the pathogenesis of atherosclerosis.

This report describes some effects of chronic minimal electrical stimulation discretely delivered to electrodes placed in the diencephalon of rabbits fed a cholesterol-rich diet. It was found that rabbits exposed to such stimulation exhibited a significantly greater degree of hyperlipemia and a much more marked atherosclerotic involvement of their aortic and coronary vasculature than did their controls.

METHODS

The present study was the result of a joint effort by individuals of the University of Oklahoma Medical Center (C. G. G.) and of the Harold Brunn Institute, Mount Zion Hospital and Medical Center, San Francisco (M. F. and S. O. B.). All physiological and anatomical procedures were done at the University of Oklahoma Medical Center, while all biochemical and pathological studies were carried out at the Harold Brunn Institute. In order to eliminate bias, all biochemical and pathological data were obtained, assessed and recorded blind, i.e., without knowledge of whether a given specimen was from an experimental or control animal.

Thirty-five male New Zealand white rabbits of the same age and comparable size from the same inbred herd were chosen at a large breeding farm. After an observation period of 1 month, 19 of these animals were operated upon under pentobarbital anesthesia. By means of a stereotaxic instrument (10), electrodes were inserted into or as close as possible to the ventral medial nucleus of the hypothalamus. The electrodes were small, bipolar, concentric and insulated from each other except at the tip. The bare electrode tips were separated from each other by a distance of 1 mm. After insertion, the electrodes and their subminiature plug attachment were secured to the calvarium with dental cement. The subcutaneous tissue and skin then were closed in layers leaving the plug outside (see Figure 1) and available for connection to either recording or stimulating apparatus.

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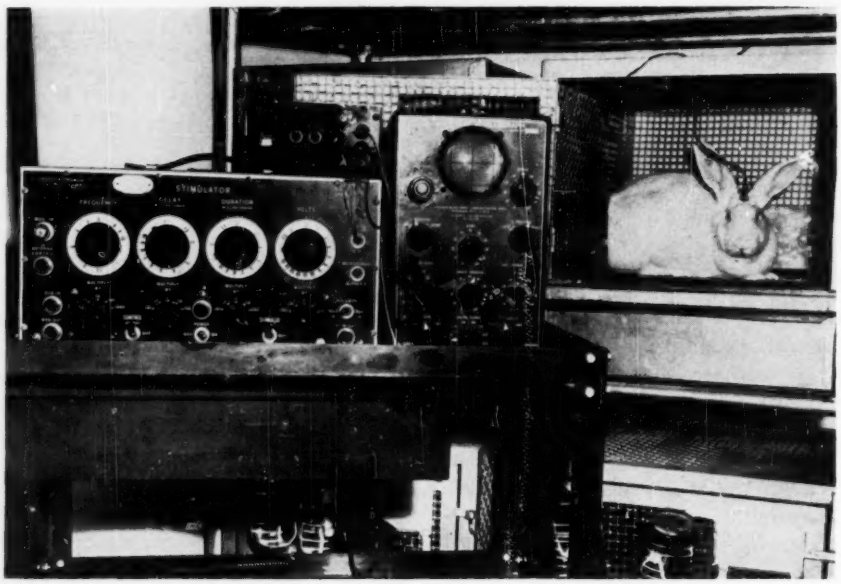


FIG. 1. RABBIT WITH IMPLANTED ELECTRODES CONNECTED TO STIMULATOR. The oscilloscope monitors (voltage or milliamperage) the parameters of stimulation. When not in use, external wires and plug are removed without touching the animal.

Following the operation, the animals were rested for 1 month. In order to determine the behavioral and autonomic thresholds, preliminary studies were done utilizing biphasic, 60 cycles per second, monitored currents of 0.2 to 0.8 ma. The current was administered at a strength of 1 to 6 v in pulses of 0.2 msec duration.

The threshold parameter (i.e., the current necessary to produce pupillary and behavioral changes) was determined for each of the 19 animals. Then they were given chronic stimulations at a current strength slightly above the determined threshold. These chronic stimulations were done four times daily, 6 days a week for 3 months. Each period or "epoch" of stimulation consisted of ten 30-second episodes of actual stimulation interspersed with ten 30-second intervals of pause. The voltage and amperage of each stimulation were monitored with an oscilloscope to ensure constancy. All stimulations were done in the cage without direct handling of the animal (see Figure 1). The daily behavior and response of each rabbit to stimulation were recorded.

Three series of animals were studied for 3 months. Series A consisted of 14 of the 19 animals bearing electrodes, that were placed on Purina rabbit chow enriched with cottonseed oil (1 per cent) and cholesterol (1 per cent) immediately following operation. Series B consisted of the remaining 5 of these 19 stimulated rabbits that were placed on Purina rabbit chow alone. Series C, a control group, consisted of 16 intact rabbits that were given the cholesterol- and oil-enriched diet.

Stimulation in the medial areas of the hypothalamus in cats has been reported to induce satiety (11), thus care was taken in the present studies to ensure comparable intake of the lipid-enriched food by all of the animals. This was accomplished by restricting the food allotment for each animal to that quantity completely consumed by those rabbits eating least. This latter amount was approximately 100 g per day containing about 160 calories.

In order to discover possible effects of acute hypothalamic stimulation, some of the animals of the above series also were studied both before and very soon after a single episode of stimulation. Thus after the first month of the experiment, seven rabbits of Series A and five of Series B were bled (20 ml) and then stimulated for two continuous "epochs." One hour later, blood samples were again obtained. For control purposes, five rabbits of Series C were bled at the same time intervals. A similar study (except that the second blood sample was obtained 5 hours after acute stimulation) was repeated upon seven rabbits of Series A at the end of 3 months. In this latter study, four rabbits of Series C also were bled at the same time intervals.

In the chronic experiment, as distinguished from the above acute experiments, blood samples were obtained before the beginning of the experimental period and then repeated each month, as indicated in Table I. In most cases, blood samples were analyzed for serum cholesterol (12), phospholipid (13), total lipid (14), and 17-hydroxycorticosteroid (15).

At the end of the 3-month period, all animals were sacrificed by intravenous injection of air. The brain was removed, fixed in formalin, cut at 50 μ and stained

TABLE I

Serum values following chronic hypothalamic stimulation

Rabbit	Prestimulation					One month					Two months					Three months					Electrode site
		Serum values (mg/100 ml)					Serum values (mg/100 ml)					Serum values (mg/100 ml)					Serum values (mg/100 ml)				
	Wt	Chol.	Phosph.	T.Lip.	17-HOCS	Wt	Chol.	Phosph.	T.Lip.	17-HOCS	Wt	Chol.	Phosph.	T.Lip.	17-HOCS	Wt	Chol.	Phosph.	T.Lip.	17-HOCS	
	kg				*μg*	*kg*				*μg*	*kg*				*μg*	*kg*				*μg*	
I. Chronically stimulated rabbits fed lipid-enriched diet																					
35*	3.48					3.50	974				3.67	647	238	1,500	6.5	3.61				22.8	VMH
37*	3.60	23				3.61	441				3.45	1,580	595	3,090	11.9	3.52				19.2	VMH
48	4.28	42	79	233	16.1	4.06	1,054	393	2,088	17.2	4.20	682	245	1,638	5.9	3.92				10.7	VMH
50	3.44	47	98	272	5.6	3.47	165	143	424	20.6	3.50	485	228	825	7.9	3.73	1,450	465	2,938	1.5	VMH
60	3.09	64	146	500	1.0	3.23	449	246	1,017		3.57	1,175	374	2,366	9.0	3.48	1,622	548	2,968	2.3	VMH
65	2.71	64	108	243	3.3	2.89	409	246	875	12.5	3.00	1,086	482	2,025	3.9	3.10	1,470	740	2,695	2.7	VMH
34*	3.63	28				3.68	769				3.46	1,225	488	2,140	10.6	3.53				22.8	AHA
47	3.28	38	82	177		3.22	574	286	1,052	11.2	3.78	720	343	1,535	21.2	3.31	1,570	666	2,802	10.7	AHA
63	3.20	93	165	434	1.0	3.29	441	266	1,080	3.9	3.68	1,048	465	2,049	1.1	3.64	1,738	630	3,155	6.9	AHA
39*	3.71	35				3.71	706				3.53	1,159	528	2,950	1.3	3.43				1.7	LHA
53	2.68	32	63	164	7.9	2.87	349	253	944	12.4	3.14	1,056	430	1,873	10.0	3.02	1,717	740	3,093	9.6	LHA
43	3.94	43	79	145		3.32	1,021	352	1,535	23.0	3.32	878	377	1,587	19.1	3.26	1,360	660	2,672	8.5	SOD
62	3.27	47	117	266	9.5	3.38	344	243	964	13.2	3.55	1,080	386	2,049	1.0	3.55	1,625	579	2,656	21.5	SOD
55	3.26	53	92	348	20.3	3.14	444	234	884	11.7	3.35	597	226	1,175	6.8	3.31	1,128	452	2,093	11.8	ARC
Av.	3.4	45	103	277	8.1	3.38	581	266	1,069	14.0	3.46	958	386	1,914	8.3	3.46	1,587	609	2,785	11.8	
SE of mean	±0.05		±9.4	±36.0	±2.3	±0.08	±69.0	±16.6	±130.0	±1.8	±0.05	±78.0	±30.0	±157.0	±1.6	±0.06	±62.0	±33	±100.0	±2.5	
II. Chronically stimulated rabbits fed regular diet																					
3	3.16	59									3.26	28				3.35	37				MesRF
7	2.80	23									3.40	18				3.60	32				MesRF
9	3.37	23									3.65	23				3.73	25				VMH
11	3.42	34									3.55	13				3.49	28				VMH
16	3.67	53									4.08	13				4.19	26				VMH
Av.	3.28	38									3.59	19				3.67	30				
III. Control rabbits fed lipid-enriched diet																					
33	3.41	11				3.53	346				3.23	374	195	897		3.32					
40	3.67	16				3.69	394				3.53	464	253	1,190		3.78				22.0	
41	4.42	28	79	159		4.29	717	299	1,482	58.6	4.30	1,105	443	2,192	33.0	4.14	1,330	562	2,198	10.7	
44	2.47	40	91	152	11.5	3.10	590	242	1,069	34.5	3.27	801	274	1,482	14.1	3.32	868	312	1,442	20.8	
46	3.69	53	94	198	15.8	3.66	562	318	1,172	17.5	3.42	785	443	1,722	10.3						
51	3.82	47	120	413	3.3	3.75	210	157	544	10.1	4.00	700	234	1,183	9.0	4.10	1,290	402	2,297	7.3	
52	3.37	48	89	259	9.1	3.34	175	173	580	1.0	3.40	500	209	992		3.17	1,935		3,620	6.1	
54	4.37	45	111	319	10.7	4.21	191	144	536	21.0	4.18	276	155	675		4.18	1,050	700	1,793	14.6	
57	3.07	26	66	269	17.5	3.11	309	198	833	14.9	3.26	558	196	1,119	14.5	3.39	1,087	339	1,796	5.0	
59	3.44	35	111	342	10.7	3.53	170	127	426	3.9	3.74	521	228	1,056	1.0	3.77	750	329	1,159	4.6	
61	3.34	48	127	398	2.3	3.38	410	273	1,035	9.5	3.68	1,385	516	2,381	1.0	3.76	1,675	573	2,159	2.3	
64	3.04	47	101	324	1.0	3.13	145	192	583	1.3	3.39	678	250	1,175	1.0	3.53	927	313	1,491	8.0	
66	3.12	39	104	348	11.9	3.08	71	112	300	19.5	3.27	362	153	722	6.2	3.53	971	307	1,651	24.0	
67	3.31	57	117	429	3.3	3.39	398	282	1,062	12.5	3.64	825	317	1,358	9.6	3.89	1,522	528	3,270	1.0	
68	3.22	49	73	232	5.6	3.19	201	134	607	1.6	3.08	500	187	949	1.0	3.16	1,242	374	2,572	1.0	
69	3.11	16	67	201	1.0	3.16	82	109	319	27.4	3.59	519	228	1,048	3.9	3.71	1,145	475	2,071	1.0	
Av.	3.43	38	96	289	8.0	3.48	311	197	754	13.4	3.56	648	284	1,259	8.7	3.65	1,215	435	2,117	9.2	
SE of mean	±0.07		±5.2	±25.0	±1.3	±0.11	±46	±18.5	±90	±2.8	±0.08	±67	±26.4	±115	±2.5	±0.08	±90	±35.6	±191	±2.1	

* These rabbits were stimulated for 3 months but were fed lipid-enriched diet for only 74 days.

with thionin to determine the loci of the implanted electrodes.

The heart, total aorta and adrenals of each animal were obtained. The aorta was first inspected grossly and graded 0 to +5 (16) for its degree of atherosclerosis. In addition, an aortic segment of 10 cm beginning at the semilunar valves was routinely obtained and analyzed for its cholesterol content.

Three cross sections of the heart (base, middle and apex) were obtained and stained with Sudan IV. Then each cross section was studied as follows: the total number of large arteries (i.e., vessels exhibiting well defined adventitia, media and intima) were counted; the total number exhibiting sudanophilic intimal hyperplasia occupying 20 per cent or more of the lumen were also counted. The percentage of the total number of large arteries in each cross section showing sudanophilia of the required degree was then calculated. A final percentage of sudanophilia for each heart was obtained by averaging the three individual percentages calculated from each of the three cross sections. Similar observations and calculations were made of the medium-sized and small coronary arteries encountered in the same three cross sections.

The adrenals were weighed. Sections also were obtained from the adrenal glands of four stimulated and three nonstimulated rabbits ingesting the lipid-enriched diet. These were stained with both Sudan IV and hematoxylin and eosin.

All animals were housed in similar cages in the same room with the temperature controlled between 61° to 71° F. Electroencephalograms were taken monthly of each stimulated animal. Blood pressure determinations (by direct arterial puncture) were obtained initially and then every month of the experimental period.

Thirty-four of the 35 rabbits survived the experimental period of 3 months.

RESULTS

A. Loci of hypothalamic stimulation. Histological examination of the brains of the 14 stimulated rabbits fed the lipid-enriched diet revealed (see Table I and Figure 2) that the locus of the stimulating electrodes was in or near the ventral medial nucleus hypothalamicus (VMH) in six rabbits, the anterior hypothalamic area (AHA) in three, the lateral hypothalamic area (LHA) in two, the supra-opticus diffusus (SOD) in two, and the nucleus arcuatus periventricularis (ARC) in the remaining rabbit.

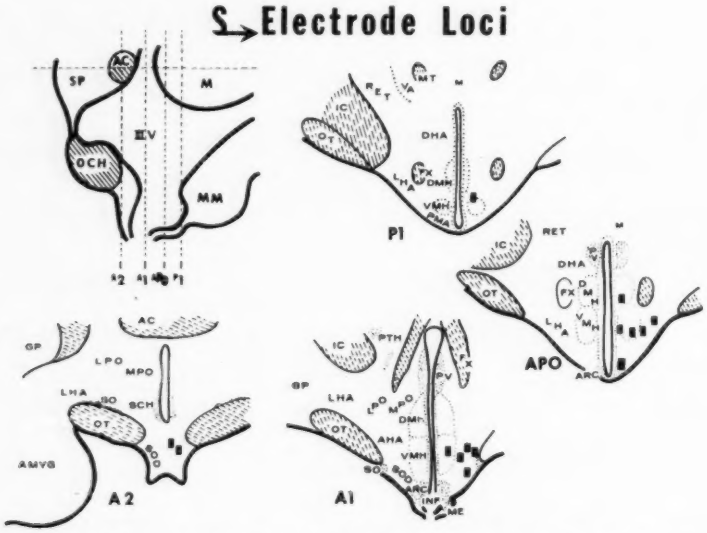


FIG. 2. DIAGRAM ILLUSTRATING THE SITE OF ELECTRODE EMPLACEMENT IN THE 14 EXPERIMENTAL RABBITS. Solid rectangles show electrode positions in the coronal-section diagrams corresponding to the planes on the midsagittal schematic (upper left-hand diagram with dashed lines showing coronal planes) through hypothalamic area (10). Coronal planes are 1 mm apart. OCH = optic chiasm; MM = mammillary bodies; III V = third ventricle; DMH = dorsal medial nucleus hypothalamus; VMH = ventral medial nucleus hypothalamicus; LHA = lateral hypothalamic area; AHA = anterior hypothalamic area; SOD = supra-opticus diffusus nucleus; ARC = nucleus arcuatus periventricularis.

Histological examination of the brains of the five stimulated rabbits fed the stock diet revealed that the locus of the stimulating electrodes was in the VMH in three of the rabbits; the locus in the remaining two rabbits was in the mesencephalic reticular formation (MesRF).

B. Behavioral and physiological observations during stimulation and thereafter. When not actually receiving stimulation, all rabbits in any of the chronically stimulated groups exhibited behavior that was identical with that of the nonoperated controls. The EEG records were in conformity with this judgment of normality of behavior between periods of stimulation, since no permanent EEG abnormality was observed in the chronically stimulated animals. The behavioral threshold to stimulation remained fairly constant for all rabbits during the total experimental period.

Nevertheless, during, and sometimes immediately after an "epoch" of stimulation marked changes in behavior could be observed in the stimulated rabbits. During stimulation almost all animals exhibited pupillary dilatation. Those rabbits whose electrodes were found embedded in the VMH, LHA or AHA exhibited rather characteristic differences in behavior from the three rabbits whose electrodes were found implanted in the SOD or ARC. The former group showed increased activity with random searching and circling in the cage; they also would display licking and chewing movements and some would ingest food avidly during and only during a phase of stimulation within an "epoch." These behavioral and gustatory changes were most marked in those animals whose electrodes were in or near the VMH. The latter group, three rabbits whose electrodes were placed in the SOD or ARC, usually displayed a decrease both in the rate and in the amplitude of respiration; moreover, they usually (but not invariably) remained totally immobile for the duration of the applied current. Rarely they also exhibited eating, licking or chewing movements either during or immediately after stimulation.

No significant chronic change in blood pressure was observed in either the experimental or the control groups given the lipid-enriched food. The average blood pressure of the control group of rabbits was 116 mm Hg (mean pressure) at the beginning of the experiment and 114 mm Hg at the end. The average blood pressure of the stimulated rabbits for the same two periods was 113 and 117 mm Hg, respectively. However, stimulation did evoke a transient rise of 5 to 20 mm Hg in five of seven rabbits tested shortly following an "epoch." It is of interest that the two rabbits that failed to show this pressor response were found to have their electrodes in either the anterior or posterior area of the medial eminence of the hypothalamus (SOD and ARC).

C. Changes in blood lipids and corticosteroids after chronic and acute hypothalamic stimulation. Despite the equivalence of age, amount of food ingested, and weight, the series of rabbits both chronically stimulated and also fed the lipid-enriched diet displayed serum cholesterol, phospholipid and total lipid values that were considerably greater throughout the 3-month period than corresponding values in the nonstimulated rabbits fed the same lipid-enriched diet (see Table I). All increases were significant, with the p value at the 0.01 level. On the other hand, rabbits that were chronically stimulated but given only the (relatively) lipid-poor stock diet (see Table I) failed to exhibit any significant change in their serum cholesterol levels.

It was of interest to us that apparently the lipid increases observed in the chronically stimulated rabbits ingesting the lipid-enriched diet appeared to be about equal in all of the rabbits, regardless of the exact site of electrode insertion. Thus, the average lipid values of the six rabbits having electrodes in their VMH were about the same during the 3-month period as those of rabbits having their electrodes in the AHA, LHA or SOD. The only exception was one animal (no. 55) whose stimulation was received in the ARC area and whose lipids after 3 months were similar to those of the controls.

A single acute stimulation, however, whether coming after either 1 or 3 months of chronic daily stimulation, failed to provoke any acute rise in the serum lipid values, irrespective of the nature of the diet (see Table II). Actually a slight fall usually was observed in the blood lipid values either at the 1 or 5 hour period after any particular stimulation. Since a similar slight fall also was observed in the nonstimulated controls, this may reflect a slight dilution of the plasma following the initial bleeding prior to stimulation.

The serum content of 17-hydroxycorticosteroids

TABLE II

Serum values following acute hypothalamic stimulation

Rabbit no.	Experimental treatment	Serum values (mg/100 ml)											
		Before treatment*				One hour after treatment				Five hours after treatment			
		Chol.	Phosph.	T.Lip.	17-HOCS	Chol.	Phosph.	T.Lp	17-HOCS	Chol.	Phosph.	T.Lip.	17-HOCS
					μg				μg				μg
		I. Rabbits fed lipid-enriched diet (1 month)											
		A. Chronically stimulated rabbits											
7	Blood sample* and stimulation	372	233	884	12.4	358	198	908	10.3				
SE of mean		±34.4	±14.3	±75	±1.95	±30	±14.2	±85.0	±2.13				
		B. Control nonoperated, nonstimulated rabbits											
11	Blood sample only	215	174	620	11.2	192	132	515	14.8				
SE of mean		±31.4	±17.1	±74.0	±2.44	±27.0	±17.5	±65.0	±5.6				
		II. Chronically stimulated rabbits fed regular diet											
5	Blood sample* and stimulation	27	69	120	22.6	19	72	124	22.1				
		III. Rabbits fed lipid-enriched diet (3 months)											
		A. Chronically stimulated rabbits											
7	Blood sample* and stimulation	920	468	1,908	15.6					884	453	1,759	13.7
SE of mean		±172	±162	±600	±3.3					±162.0	±68.0	±570.0	±3.4
		B. Control nonoperated, nonstimulated rabbits											
4	Blood sample only	740	326	1,294	17.8					671	294	1,358	14.4

* All rabbits bled 3 to 7 hours before acute stimulation.

failed to be influenced by either chronic (see Table I) or acute (see Table II) stimulation in either of the two stimulated series.

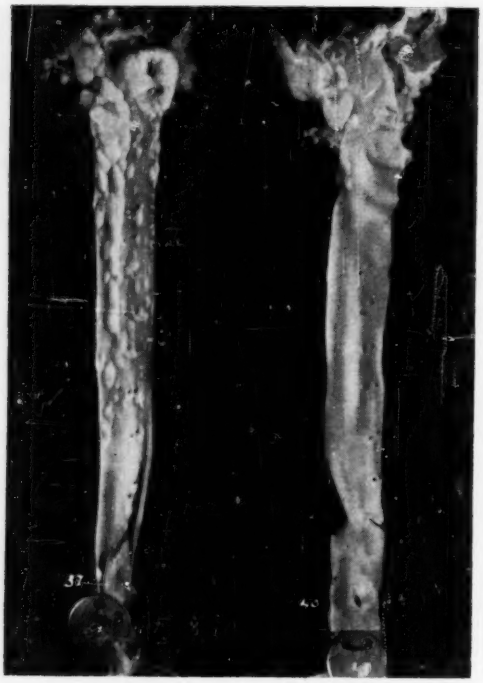
D. Pathological changes after chronic hypothalamic stimulation. No difference in heart size was observed in any of the three groups. However, striking differences in the degree of aortic atherosclerosis were exhibited by the chronically stimulated rabbits fed the lipid-enriched diet as compared with the atherosclerosis shown by control nonstimulated rabbits fed the same quantities of the same diet (see Table III and Figure 3). Thus eight of the 14 stimulated rabbits showed moderate to marked aortic atherosclerosis (grade 2 or above), whereas only two of the 15 control animals showed a similar degree of involvement

TABLE III

Vascular and adrenal changes following chronic hypothalamic stimulation

Rabbit no.	Electrode site	Aorta: Gross grading	Aorta: Cholesterol	Per cent atherosclerotic involvement coronary vasculature: Large	Per cent atherosclerotic involvement coronary vasculature: Med. and small	Adrenal wt
			g/100 g			*mg*
I. Chronically stimulated rabbits fed lipid-enriched diet						
35*	VMH	3.5	3.30	0	38	910
37*	VMH	5.0	7.37	4[illegible]	14	750
48	VMH	3.0	4.75	25	45	968
50	VMH	3.0	5.52	50	37	1,205
60	VMH	5.0	11.55	12	40	1,145
65	VMH	1.0	8.17	75	42	2,145
34*	AHA	3.0	1.88	29	75	1,000
47	AHA	1.0	2.96	10	43	1,452
63	AHA	1.0	3.56	66	48	800
39*	LHA	3.0	2.69	14	54	975
53	LHA	1.5	3.74	71	35	1,285
43	SOD	1.0	2.02	57	33	1,040
62	SOD	2.0	4.49	20	54	1,000
55	ARC	1.0	2.83	0	10	1,318
Average		2.4	4.63	34	41	1,142
SE of mean		±0.37	±0.70	±6.8	±4.1	±84.0
II. Control nonstimulated rabbits fed lipid-enriched diet						
33		0.5	0.45	25	33	1,235
40		1.0	1.66	25	17	1,150
41		4.0	3.28	22	32	1,570
44		0.5	1.61	30	42	1,035
51		1.0	2.90	33	35	930
52		1.0	2.85	0	24	1,620
54		0.5	2.56	0	20	1,010
57		2.5	3.72	0	15	1,473
59		0.5	2.05	33	15	785
61		1.0	4.12	67	39	685
64		0.5	1.09	0	15	745
66		0.0	1.00	0	17	1,265
67		0.0	0.90	40	50	865
68		0.5	0.72	0	18	995
69		0.0	1.03	0	14	910
Average		0.9	2.0	18	26	1,085
SE of mean		±0.26	±0.29	±5.1	±2.9	±91.0
III. Chronically stimulated rabbits fed regular diet						
3	MesRF	0.0	0.35	0	0	380
7	MesRF	0.0	0.30	0	0	600
9	VMH	0.0	0.42	0	0	475
11	VMH	0.0	0.22	0	0	700
16	VMH	0.0	0.38	0	0	600
Average		0.0	0.33	0	0	551

* These rabbits received lipid-enriched diet for only 74 days.



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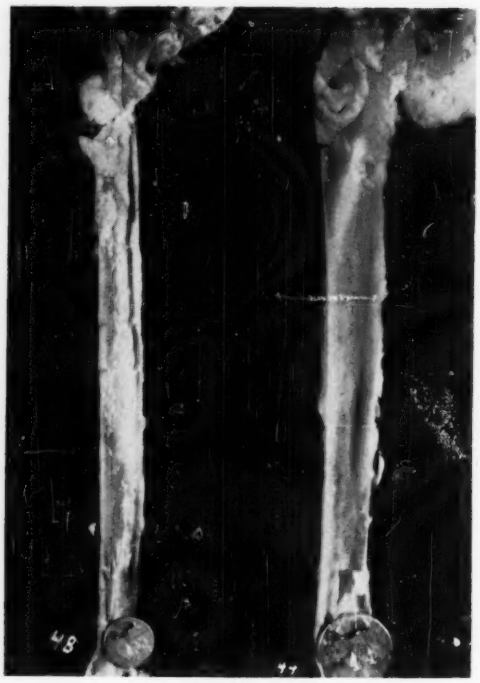

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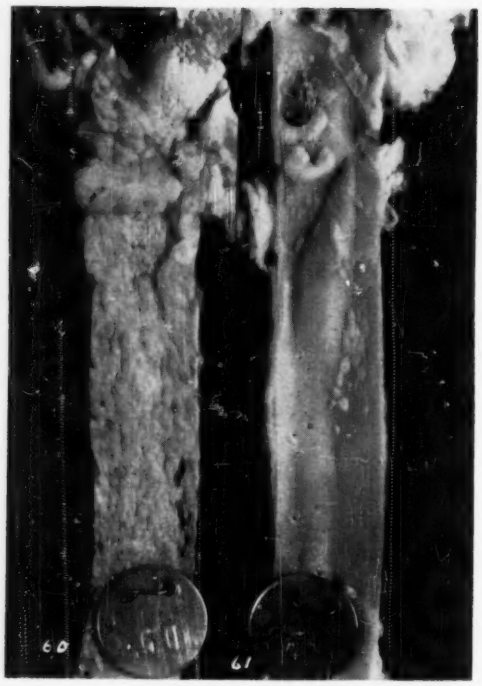

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FIG. 3. AORTAS OF STIMULATED AND CONTROL NONSTIMULATED RABBITS FED CHOLESTEROL. The aorta on the left of each photograph is that of a stimulated rabbit, and the one on the right, of a nonstimulated rabbit of the same experiment. The greater degree of atherosclerotic involvement of the aortas of the stimulated rabbits can easily be seen.

(p value significant at the 0.01 level). This difference observed by gross inspection was corroborated by analysis of the aortic segments for cholesterol content. Thus the average cholesterol concentration (4.63 g per 100 g of dry weight) of the 14 aortic segments of the stimulated rabbits was over twice that (2.0 g per 100 g of dry weight) found in the control nonstimulated rabbits ingesting the same diet (p value significant at 0.01 level). By the same token (see Table III), a greater percentage of the coronary vasculature of the stimulated rabbits was atherosclerotic as compared with the vasculature of the control group.

A comparison of the degree of aortic atherosclerosis with the diencephalic site of electrode emplacement strongly suggests that the animals receiving stimulation in or in the vicinity of the VMH had excess aortic atherosclerosis and cholesterol infiltration. Thus the average grade of aortic atherosclerosis found in these six animals was 3.3 as compared to the grade of 2.4 for the total group of experimental animals, and the average cholesterol content of their aortic segments was 6.76 g per 100 g dry weight as compared to 4.63 g for all experimental animals and 2.0 g for the controls. The relatively small number of animals studied, however, precludes the drawing of an absolute conclusion concerning the significance of this difference.

On the other hand, as Table III indicates, stimulation of the group of five rabbits that were not fed the lipid-enriched food failed to induce either grossly observed or chemically detected atherosclerosis.

We were unable to detect any histological difference in the adrenals of either the stimulated or the unstimulated rabbits fed the lipid-enriched diet. In both groups, the adrenals (see Table III) were greatly and, on the average, equally enlarged. This increase in size and weight appeared to be due to lipid and cholesterol deposition.

DISCUSSION

The preceding data appear to indicate that rabbits fed a diet excessive in cholesterol and cottonseed oil and, in addition, receiving chronic stimulation of their diencephalon in the vicinity of the ventral medial nucleus, the lateral and the anterior hypothalamic areas, will exhibit a greater degree of chronic lipemia and a greater degree of aortic and coronary atherosclerosis than their nonstimulated controls. Although we were impressed by the fact that apparently the highest degree of atherosclerosis was exhibited by the six animals that were found to have their stimulating electrodes fixed in the ventral medial nucleus, the small number of rabbits studied makes the conclusion of an association between atherosclerosis and stimulation of the ventral medial nucleus a tentative conclusion at best.

The mechanism(s) whereby this chronic stimulation may have brought about the observed changes in the arterial vessel walls remains to be elucidated. However, it seems clear from the above study that they were not mediated by changes in food intake, by *chronic* changes in blood pressure or in physical activity. Our failure to observe any change in the corticosteroid level of the serum either during the period of chronic stimulation or soon (1 to 5 hours) after acute stimulation suggests but does not prove that the adrenal glands of these rabbits did not play a very important role in the pathogenesis of the atherosclerosis found.

Perhaps the chronically sustained greater degree of lipemia observed in the stimulated animals was responsible for the more extensive aortic and coronary atherosclerosis found. Certainly this lipemia must have a significant role if only because identical hypothalamic stimulation of rabbits given ordinary rabbit laboratory chow failed to elicit any detectable change either in the blood lipids or in the arterial walls. The means whereby chronic diencephalic stimulation (under the conditions of the present study) might intensify the hyperlipemic response of rabbits fed controlled rations of lipid-enriched food also remains to be understood.

SUMMARY

Evidence has been presented consistent with the hypothesis that chronic electrical stimulation delivered to certain hypothalamic areas significantly intensifies both the rate of increase and the amount of hyperlipemia in rabbits fed a lipid-enriched diet. Such animals also showed a much greater atherosclerotic involvement of their aortic and coronary vasculature than did control animals given a similar diet. Those rabbits receiving

stimulation in or near the ventral medial nucleus appeared to exhibit no greater hyperlipemia but considerably more atherosclerosis than the animals stimulated elsewhere.

It is concluded that central nervous system mechanisms exist which, under certain conditions, are capable of significantly influencing arterial atherogenesis.

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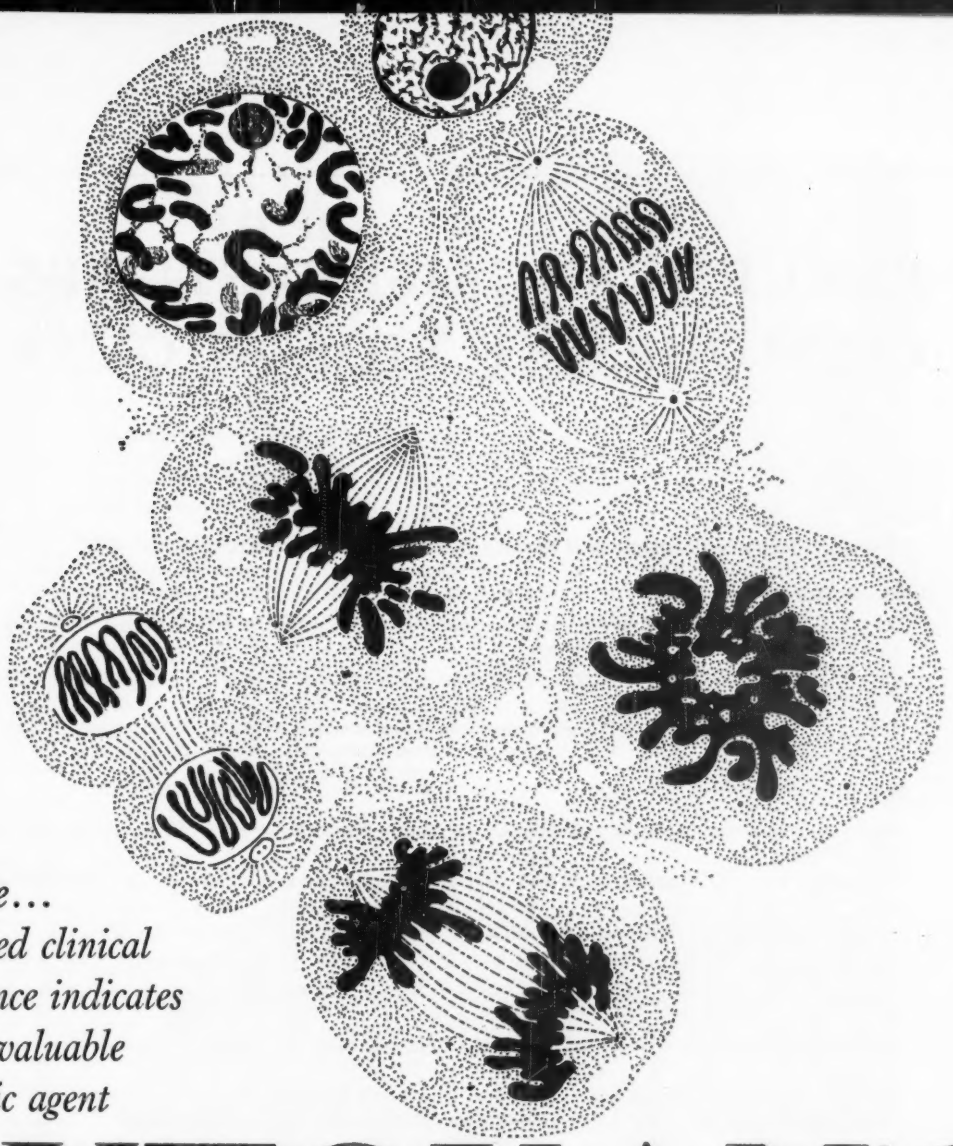

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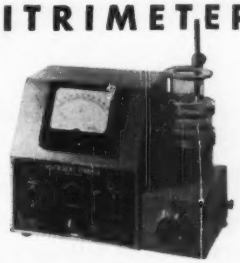

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Volume 19, No. 4.

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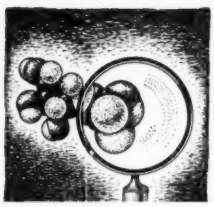
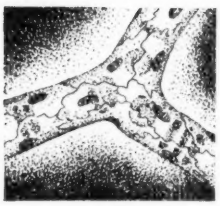
IT HAPPENED THIS MONTH...

a glance at yesterday in relation to today



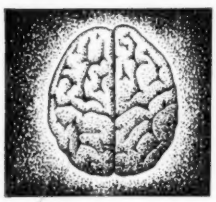
IN DECEMBER – (1926) – proof of the chemical configuration of thyroxine is completed. Earlier in the year C. R. Harington had shown that thyroxine is a tetraiodo-derivative of the *p*-hydroxyphenyl ether of tyrosine. Now, he and Barger[1] have produced a synthetic thyroxine which is chemically and physiologically identical with the natural hormone. Evidently thyroxine is not – as had previously been suggested – a metabolic derivative of tryptophane, but is formed by the coupling of two molecules of diiodotyrosine.

Today American and European biochemists are still actively studying the metabolic transformations of the thyroid hormones. There is promise here of important medical applications. Here, as in other areas of amino acid metabolism, isotope-labeled amino acids are important research tools. Schwarz BioResearch supplies a long list of biologically important amino acids labeled with C^{14}, S^{35}, N^{15}, and O^{18}.



IN DECEMBER – (1936) – Schultze and Elvehjem[2] report some interesting changes in the blood glutathione of animals with nutritional anemia. Erythrocytes from anemic rats show a decrease in total glutathione with a marked shift to the oxidized form. Consequently, there is a great decrease in reduced glutathione. On the other hand, there is an increase in both the total and reduced glutathione content of pig erythrocytes as anemia becomes severe.

If you are interested in studying the function of glutathione in health and disease, Schwarz BioResearch can supply you with both the oxidized and reduced forms – plain or labeled with S^{35} or C^{14}. We can also provide you with the common sulfur amino acids and many of their derivatives.



IN DECEMBER – (1956) – the Journal of Neurochemistry carries a paper on the nucleoside requirements of the perfused brain. In the absence of the liver, aerobic glycolysis soon develops in the brain and impermeability to glucose follows. Small amounts of cytidine and uridine added to the perfusion fluid help to maintain normal carbohydrate metabolism for four hours or longer without the liver being present in the circulatory system. It is suggested that these two nucleosides may exert this "protective" action by preventing accumulation of excessive amounts of lactic acid.[3]

Neurochemistry is only one field of investigation where nucleosides are playing an increasingly important role. Research workers the world over rely on Schwarz for purine and pyrimidine nucleosides, isotopically stable or labeled with C^{14} or H^3. Write us about your requirements for nucleic acid derivatives and other compounds.

You are invited to visit us at Booth 96 at this month's AAAS meeting.

1. Harington, C. R., and Barger, G.: Chemistry of thyroxine. III. Constitution and synthesis of thyroxine. Biochem. J., *21*:169 (1927). 2. Schultze, M. O., and Elvehjem, C. A.: Studies on the glutathione content of the blood in nutritional anemia. J. Biol. Chem. *116*:711 (Dec.) 1936. 3. Geiger, A., and Yamasaki, S.: Cytidine and uridine requirement of the brain. J. Neurochemistry *1*:93 (Dec.) 1956.

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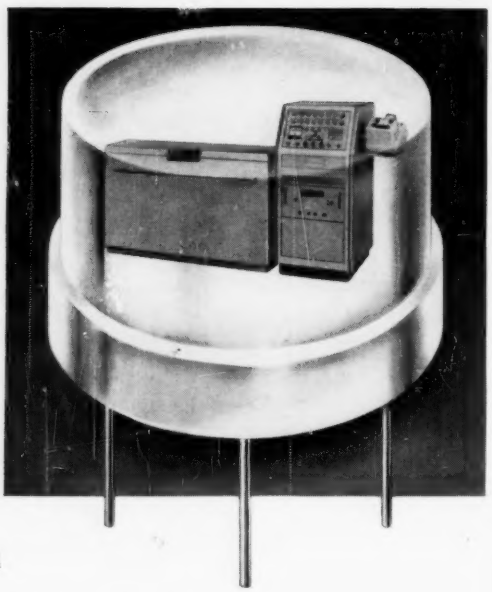

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